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13. ABSTRACT (Maximum 200 words) Effects of oxidative stress (OS) on nuclear signal transduction and on cellular anti-oxidant mechanisms, were compared in human blood lymphocytes. DNA-binding activities of 3 transcription factors (TF): NFκB, AP-1 and NFAT, were abolished in activated T lymphocytes by all types of OS. Radiation exposure was also studied in B cells and had the same effect as on T cells. Only exposure to the lowest H ₂ O ₂ concentration induced lipid peroxidation. Enhanced catalase activity was detected upon exposure to oxidative stress. Glutathione peroxidase activity and reduced glutathione, vitamin C and vitamin E levels, were not modulated by oxidative stress. N-acetyl cysteine protected human lymphocytes against oxidative stress. Conclusions: 1. Suppression of specific TF functions, and IL-2 biosynthesis, can serve as a marker of lymphocyte exposure to OS; 2. Based on these results, an <i>in vitro</i> screening assay to identify oxidants, and an assay to monitor human exposures to oxidants, can be developed. 3. Enhanced catalase activity may be developed as a marker for the ability of cells from exposed soldiers to mount an anti-oxidative response. 4. N-acetyl cysteine was identified as a compound that may protect individuals exposed to oxidants.				
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FOREWORD

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TABLE OF CONTENTS

	PAGE
FRONT COVER	1
SF 298	2
FOREWORD	3
TABLE OF CONTENTS	4
INTRODUCTION	5
BODY	7
CONCLUSIONS	24
REFERENCES	25
BIBLIOGRAPHY SUPPORTED BY THIS GRANT	29
LIST OF PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT	31
APPENDICES	32

INTRODUCTION

Lymphocyte activation and signal transmission. T lymphocytes are activated following the binding of a ligand to the antigen receptor complex (CD3/Ti). One of the early manifestations of this interaction is the transcriptional activation of the IL-2 gene¹ which requires simultaneous activation of protein kinase C and elevation of intracellular Ca^{2+} concentration². IL-2 is a pivotal lymphokine involved in B and T lymphocyte, as well as natural killer cell, regulation¹. An immediate consequence of T cell activation is the phosphorylation of a wide range of proteins^{3,4}. The modulation of IL-2 transcription by nuclear factor(s) can serve as a general readout that would be affected by any abnormality occurring earlier in the pathway. The promoter region of the IL-2 gene, in the 5' flanking region, controls induced T cell specific gene expression⁵. A transcriptional enhancer lies in this region and responds to signals generated after activation through the T cell antigen receptor⁶.

A number of positive regulatory elements have been identified in this region, including: NFAT, AP-1, NFkB and EGR-1. The activities of proteins capable of binding to these sequences and thereby enhancing the expression of the IL-2 gene (transcription factors), appear only in activated human T cells⁷⁻⁹. Other transcription factors, expressed in human T lymphocytes, bind to the following sequences in the IL-2 promoter: AP-3, Oct-1, and Sp1. NFAT-1, AP-1 and NFkB, which are only expressed in primary human T cells following stimulation, are plausible targets for suppression of T cell activation. A zinc finger containing protein that inhibits IL-2 gene expression has also been described. A nucleotide sequence (NRE-A) that binds to this protein has been identified between -101 and -110¹⁰.

B lymphocytes do not express the IL-2 gene but do exhibit activities of some of the transcription factors that regulate the expression of IL-2 in T cells. In B cells, these transcription factors are involved in the enhancement of the expression of other genes such as immunoglobulin genes. B cells express Oct-1, NFkB and AP-1 activities¹¹⁻¹³. NFAT DNA-binding activity can also be detected in B cells¹³ although it does not appear to enhance gene expression in human B cells¹⁴.

Environmental oxidative stress. A variety of distinct biochemical changes in lymphocytes and in various other target cells are induced by oxidants, e.g., hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^\bullet). These changes include alterations in enzymatic activities, lipid peroxidation and damage to DNA. H_2O_2 rapidly permeates cells and would, in most cellular environments, have a lifetime that would permit it to diffuse appreciable distances before reaction. It is therefore proposed that H_2O_2 is the dominant oxidant leading to DNA strand breaks¹⁵. Also, oxidizing reactive species on free-radical-damaged proteins, protein hydroperoxides that can consume glutathione, have been demonstrated. The long-lived nature of the reactive moieties indicates that they may be able to diffuse and transfer damaging reactions to distant cellular sites¹⁶.

The principal oxidants in the lower atmosphere are ozone and two by-products of ozone photodissociation, the hydroxyl radical and hydrogen peroxide¹⁷. Ozone is a very toxic air pollutant affecting organic molecules via free radical- and lipid peroxide-mediated mechanisms¹⁸. T lymphocytes from subjects exposed to ozone *in vivo* exhibited significantly reduced mitogenic response for several weeks following

the exposure^{19,20}. Since ozone is a highly reactive oxidant pollutant, it is unlikely that it interacts directly with blood lymphocytes. Rather, the lymphocytotoxic effect of ozone is probably mediated by soluble products of pulmonary cells that are affected directly by the oxidant²¹. Pryor et al.²² have demonstrated that the interaction of ozone in the presence of water with unsaturated fatty acids, ozone's primary target in lung lining fluids, produces aldehydes and hydrogen peroxide. When bronchoalveolar lavage (BAL) was used, the yield of hydrogen peroxide production was 55%. Based upon those experiments with ozone at 2.8 ppm (nearing smog levels) the interaction with BAL would yield approximately 8.25 nmoles/ml H_2O_2 over two hours. The polyamine oxidase system we studied involves oxidative stress exerted on lymphocytes by enzymatic products, which include hydrogen peroxide and an aldehyde, at about 5 nmoles/ml H_2O_2 over forty eight hours. This exposure approximates the daily average of urban ozone profile including night time - 0.1 ppm²³. Therefore, we propose that our system can serve as a model for environmental oxidant exposure.

Ionizing radiation can be used as a means of introducing oxygenating radicals into lymphocytes in a geometrically and temporally precise way. The absorption of radiation involves splitting H_2O molecules (the most common constituent of cells) into OH^\bullet and H^\bullet radicals which are initially distributed in proportion to the radiation dose distribution²⁴. OH^\bullet radicals generated within a cell would generally react immediately with very little diffusion into the surrounding medium. In addition, irradiation of dissolved O_2 will produce the superoxide radical, HO_2^\bullet , also following the radiation dose distribution. The superoxide radical has intermediate reactivity between that of OH^\bullet and H_2O_2 and will diffuse moderate distances before reacting. In comparison, H_2O_2 is less active and may diffuse many cell diameters before interacting with cellular macromolecules. Accelerated electrons are quite easy to control and precise levels of oxidative stress can be generated by irradiating cells and the surrounding medium. A radiation dose of 1.0 Gy will generate 2.72×10^{-8} mol/l of OH^\bullet , 0.68×10^{-8} mol/l of H_2O_2 and 0.008×10^{-8} mol/l of HO_2^\bullet in water, and similar values are expected initially in the water component of cells growing in nutrient medium. The use of ionizing radiation to administer oxidative stress enhances the control of the concentration and timing of the exposure and provides a different type of stress (mainly mediated by OH^\bullet) than that produced by enzymatically-generated H_2O_2 .

All respiring cells produce H_2O_2 and have various intracellular anti-oxidant mechanisms. Most of the H_2O_2 is reduced to H_2O by two enzymes: catalase and selenium-dependent glutathione peroxidase²⁵. In addition, intra- and extra-cellular organic molecules are protected from oxidation by various anti-oxidants that can also be used pharmacologically and nutritionally to control oxidative damage. Oxidants present in the gas phase of cigarette smoke cause lipid and protein peroxidation. N-acetylcysteine (NAC), that can scavenge several oxidant species including H_2O_2 ²⁶, increased (when given orally) glutathione plasma levels and was therefore suggested to have beneficial potential in smokers²⁷. Vitamins C and glutathione appear to be important in protecting plasma lipids and proteins against oxidant stress, respectively^{28,29}.

Polyamine oxidation and IL-2 biosynthesis. We have previously described a new mechanism of IL-2 down-regulation³⁰. Endogenous H₂O₂ produced by monocytes and endogenously produced or exogenously added polyamines all provide down-regulatory signals for IL-2 production by human peripheral blood T cells. The interaction between polyamine oxidase (PAO) and the polyamine spermidine generates products (including H₂O₂) that suppress IL-2 production. PAO activity suppressed protein tyrosine phosphorylation and calcium mobilization. If indeed the effects of PAO on early signaling events cause reduced IL-2 biosynthesis then we would expect to detect those effects later in the signaling cascade, reflected in the expression of transcription factors that regulate the IL-2 gene.

Hypothesis

1) Oxidative stress alters the expression of transcription factors in human T lymphocytes; 2) The effects of oxidative stress on human lymphocytes are modulated by endogenous mechanisms and can be reversed by exogenous therapeutic measures.

Technical objectives

To study three models of inducing oxidative stress in lymphocytes: a) PAO activity generating extra-cellularly low levels of H₂O₂ for two days (mimicking exposures to environmental chemical toxicants); b) electron irradiation generating both extra- and intra-cellularly mainly OH[•]; and c) high levels of reagent H₂O₂ generating short but acute stress.

The following questions were asked:

Do human lymphocytes subjected to oxidative stress: a) exhibit abnormal cellular function-expression of transcription factors that regulate the interleukin 2 gene (essential for T lymphocyte function)?; and b) sustain damage as assessed by an independent and established method: measurement of lipid peroxidation?

To what extent, if at all, are levels of endogenous antioxidant molecules (catalase, glutathione peroxidase, glutathione, vitamins E and C) altered in human T lymphocytes subjected to oxidative stress?

Do aspirin and N-acetyl cysteine protect human T lymphocytes against oxidative stress?

BODY

Methods

T and B cells from the peripheral blood of healthy donors were studied. Cells were incubated for all assays in a serum-free medium, because fetal calf serum contains PAO activity³⁰. Therefore, RPMI-1640 with Nutridoma-HU supplement (Boehringer Mannheim Biochemicals, Indianapolis, IN) was used.

Lymphocyte preparation. Heparinized peripheral blood from healthy donors was used as a source of lymphocytes. Cells were purified by Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation. The resultant mononuclear cell preparation was allowed to adhere to plastic dishes to

remove macrophages and other adherent cells. Non-adherent mononuclear cells were then used either as a source of T or B cells.

For T cells, non-adherent mononuclear cells were mixed with a suspension of neuroaminidase-treated sheep erythrocytes and incubated at 37°C for 15 min, followed by centrifugation and further incubation at 4°C for 45 min. Thereafter, the rosetted cells were obtained by centrifugation through Ficoll-Hypaque. The erythrocytes in the cell pellet were lysed by exposure to 0.83% NH₄Cl. The rosetted cells contained more than 98% CD3⁺ T cells, and 0.4-1% M3⁺ monocytes as determined by flow cytometry.

For B cells, non-adherent mononuclear cells were mixed with magnetic beads carrying anti-B cell antibodies on their surface and incubated at 4°C for 15 minutes. Dynabeads M-450 with anti-CD19 antibodies (DynaL, Lake success, NY) were used at ten million beads/ml. The rosetted CD19⁺ B cells were isolated by magnetic force and the beads were detached using reagents and equipment from Dynal. The resultant isolated B cells were un-stimulated and 99% pure, as determined by flow cytometry.

Oxidative stress. These modes were used:

- a) A longitudinal low level extra-cellular stress (mimicking exposures to environmental chemical toxicants) - lymphocytes were pre-incubated for 2 days with a commercial preparation of PAO (Sigma) at 5×10^{-4} U/ml and spermidine at 5 μ M. This exposure generates gradually 5 μ M H₂O₂ over two days³⁰.
- b) Electron radiation generating both extra- and intra-cellularly mainly OH[•] - lymphocytes were exposed to a radiation dose of 6 Gy for 5 minutes. This dose produces non-lethal cellular responses and generates oxidants per time unit at about 20 fold higher levels than mode a), but for a much shorter period of time. We used a 2.5 MeV Van de Graaff accelerator that is capable of generating electrons or protons to a maximum energy of 2.5 MeV. Doses were continuously monitored by means of parallel plate ionization chambers coupled with a stable, vibrating reed, electrometer.
- c) A short high level extra-cellular stress-reagent H₂O₂ was added directly at 20, 50, 100 and 200 μ M for 2 hours.

The suppressive effect was studied in the pre-incubation mode in order to exclude any possible effects on the assay used. All reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Measurements of transcription factor activities. For T cell activation we used PHA (1 μ g/ml) + TPA (5 ng/ml), and for B cell activation we used anti- μ antibodies (Pharmingen, San Diego, CA; pre-coated on the bottom of 24-well plates), for 6 h at 37°C, 5% CO₂, prior to collecting the cells for nuclear extraction.

DNA-binding

Electrophoretic mobility shift assay (EMSA)

Preparation of nuclear extracts. This method is suitable for small numbers of cells and therefore appropriate (based on our experience, Flescher et al.³¹) for studies of peripheral blood lymphocytes. Cells were washed and resuspended in Tris buffered saline, transferred to an Eppendorf tube and repelleted. The cell pellet was resuspended in a buffer containing: 10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 5 μ g/ml Aprotinin, 5 μ g/ml Antipain, 100 μ M Benzamidin, 5 μ g/ml Leupeptin, 5 μ g/ml Pepstatin, 5 μ g/ml soybean trypsin chymotrypsin inhibitor, pH 7.9. The cells were allowed to swell on ice for 15 min and NP-40 at 0.625% was added. The tube was vortexed for 10 seconds and

centrifuged for 30 seconds in a microfuge. The nuclear pellet was resuspended in a buffer containing 20 mM Hepes, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and the 7 aforementioned protease inhibitors, pH 7.9. The tube was vigorously rocked on wet ice for 15 minutes on a shaking platform and the nuclear extract was centrifuged for 5 minutes. Protein concentration of the supernatant was determined (Bradford method, Bio-Rad Protein Assay Kit). Aliquots were stored at -70°C .

DNA-protein interactions. DNA probes containing the binding sites from the IL-2 promoter region were purchased from Genosys (The Woodlands, TX). The probe for NFAT-1 spans between nucleotides -255 and -285:

5'-GGAGGAAAACTGTTTCATACAGAAGGCGTT-3'

The probe for AP-1 spans between nucleotides -140 and -156:

5'-TTCAAAGAGTCATCAG-3'

The probe for NF κ B spans between nucleotides -190 and -214:

5'-TAACAAACAGGGATTTCACCTACAT-3'

The probe for AP-3 spans between nucleotides -201 and -211:

5'-AAAGAGGGATT-3'

The probe for IL-2 transcription inhibitory protein (NRE-A) spans between nucleotides -99 and -112:

5'-AATTCCAGACAGGTAAAGTGTTAA-3'

The probes were labeled with ^{32}P -ATP using T4 polynucleotide kinase (Promega). We combined 10,000 cpm DNA probe (~ 0.2 ng), 2 μg poly(dI-dC) (a nonspecific competitor DNA), 3 μg BSA (a protein carrier) and 10 μg nuclear extract in a final reaction volume of 20 μl . The binding reaction mixture was incubated for 15 min in a 30°C water bath. The protein-DNA complexes were detected on a 4% low-ionic-strength native polyacrylamide gel. The gel was dried under vacuum and autoradiographed.

Measurements of lipid peroxidation

Quantitative peroxide assay. A lipid compatible formulation of the PeroXOquant Quantitative Peroxide Assay (Pierce Chemical Co., Rockford, IL) was used. This assay is adapted to measure cellular hydroperoxides. To differentiate between hydrogen peroxide and peroxides of cellular molecules (such as lipid peroxides) we followed the recommendations of the manufacturer and regarded any catalase (7000U/ml)-inhibitable measurement as representing hydrogen peroxide. In the assay, peroxides convert Fe^{2+} to Fe^{3+} in a sulfuric acid solution. The Fe^{3+} complexes with the xylenol orange dye to yield a purple product with absorbance at 540-600 nm. The molar extinction coefficient of the xylenol orange- Fe^{3+} complex is $1.5 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ in 25 mM H_2SO_4 at room temperature. Five million cells were lysed by sonication (two 10 seconds pulses with a 10 seconds interval) and incubated for 15-20 minutes at room temperature in the following working solution (10 times the volume of the sonicate): 0.25mM ammonium ferrous (II) sulfate, 25mM H_2SO_4 , 4mM BHT, 125 μM xylenol orange in methanol. Results were read at 595 nm in a microtiter plate reader. For calibration and validation, a series of hydrogen peroxide solutions at concentrations between 1 μM to 1mM were prepared and assayed. Results were calculated per protein concentrations as determined by the Bradford method. Since the method allows to measure peroxides without lipid extraction, a blank without ammonium ferrous (II) sulfate and H_2SO_4 was used to subtract endogenous iron, and other transition metals, interferences.

Measurements of catalase activity. Ten million cells were lysed by sonication (two 10 seconds pulses with a 10 second interval) in 0.5 ml PBS. The

resultant sonicate was centrifuged at 14,000xg for 10 minutes at 4°C. Catalase activity was measured in the supernatant. Fifty µL of the supernatant were mixed with 600 µL of 15 mM H₂O₂ in a cuvette. The kinetics of the decrease in light absorbance at 240 nm (H₂O₂ decomposition) were determined for 3 minutes in a DU 640 spectrophotometer (Beckman, Fullerton, CA). A cuvette containing only PBS served as blank. A cuvette without a sample was used to ensure that H₂O₂ does not decompose spontaneously under our experimental conditions. Enzymatic activity was expressed as the rate constant of a first-order reaction (k) divided by the protein concentration. A₁ and A₂ refer to the absorbance before and after a given time interval of measurement (t), respectively. $k = (2.3/t)(\log A_1/A_2)(\text{sec}^{-1} \cdot \text{mg protein}^{-1})$ ^{32,33}.

Glutathione peroxidase assay. Ten million cells were lysed by sonication (two 10 seconds pulses with a 10 second interval) in 0.5 ml PBS. The resultant sonicate was centrifuged at 14,000xg for 10 minutes at 4°C. Two hundred µL of cell lysate were added to an equal volume of double-strength Drabkin's reagent (1.6 mM KCN, 1.2 mM K₃Fe(CN)₆, 23.8 mM NaHCO₃) and the mixture was agitated for several seconds. Aliquots were added to 2.66 ml 50 mM sodium phosphate/5 mM EDTA buffer, pH 7.5. A one to one dilution of Drabkin's reagent in water was used as a blank. The following were added sequentially: 0.1 ml 8.5 mM NADPH, 1 unit of glutathione reductase (type III, Sigma), 10 µl 1.125 M NaN₃, 0.1 ml 0.15 M glutathione, and 0.1 ml 2.2 mM H₂O₂. The changes in absorbance at 340 nm were determined over 5 minutes. Activity was expressed as nmol of H₂O₂ converted/mg protein/min³⁴.

Reduced Glutathione assay. Glutathione was assayed with the Bioxytech GSH-400 kit by Oxis International (Portland, OR). This kit determines glutathione specifically. Thirty five million cells were lysed in 0.5 ml of ice-cold 5% metaphosphoric acid. The lysate was centrifuged at 1000xg for 10 minutes at 4°C. Three hundred µL of the supernatant were mixed with 0.6 ml of the following: 200 mM potassium phosphate, pH 7.8, containing 0.2 mM diethylenetriaminepentaacetic acid (DTPA) and 0.025% lubrol. Fifty µL of 12 mM 4-chloro-1-methyl-7-trifluoromethyl-quinolinium methylsulfate in 0.2N HCl were added to the above mixture and the tube was vortexed. Fifty µL of 30% NaOH were added and the mixture was vortexed. Samples were incubated at room temperature for 10 minutes in the dark and absorbance was read at 400 nm. A standard curve was established with reduced glutathione at 0-100 µM.

Vitamin C assay. Fifty million cells were lysed in 1.2 ml 3% perchloric acid. The lysate was vortexed and centrifuged at 20,000xg for 20 minutes at 4°C. The concentration of Vitamin C in the supernatant was determined by high pressure liquid chromatography at Lab Corp., Special Chemistry Division, Burlington, NC.

Vitamin E assay. Fifty million cells were resuspended in 830 µl PBS. The cell suspension was mixed with 170 µl of 25% ascorbic acid. The sample was heated for 5 minutes in a 70°C water bath. One hundred and seventy µl of 10N KOH were

added and the mixture was mixed and heated for 30 min. at 70°C. The sample was cooled in an ice bath. Four ml of hexane were added, the mixture was shaken vigorously for 2 min and centrifuged at 1500 rpm for 5 minutes at 4°C. The upper layer (hexane phase) was separated and fluorescence was determined at excitation 298nm, emission 328nm. Vitamin E levels were calculated with a standard curve of 0-10 µg/ml hexane³⁵.

IL-2 measurement. Human IL-2 was measured by a two-antibody (Genzyme Duoset) ELISA using biotin-streptavidin-peroxidase detection. Polystyrene plates (Maxisorb, Nunc) were coated with capture antibody in PBS overnight at 25°C. The plates were washed 4 times with 50mM Tris, 0.2% Tween-20, pH 7.0-7.5 and then blocked for 90 minutes at 25°C with assay buffer (PBS containing 4% BSA and 0.01% Thimerosal, pH 7.2-7.4). The plates were washed 4 times and 50µL assay buffer was added to each along with 50µL of sample or standard prepared in assay buffer and incubated at 37°C for 2 hours. The plates were washed 4 times and 100µL of biotinylated detecting antibody in assay buffer was added and incubated for 1 hr at 25°C. After washing the plate 4 times, streptavidin-peroxidase-polymer (RDI) was added and incubated at 25°C for 30min. The plate was washed 4 times and 100µL of substrate (TMB, Dako) was added and incubated at 25°C for approximately 10-30 min. The reaction was stopped with 100µL 2N HCl and the OD at 450nm (minus OD at 650nm) was read on a microplate reader (Molecular Dynamics). A curve was fit to the human IL-2 standards (Genzyme Duoset, 3.9-500 pg/ml) and IL-2 concentration in each sample was calculated from the standard curve equation. An internal control containing 50 pg/ml IL-2 was analyzed in parallel.

Statistical analysis of data. Data was analyzed, where appropriate, using student's T test.

Results

Our Statement Of Work proposed to answer the following questions: 1) Does oxidative stress induce abnormal cellular function-expression of transcription factors regulating the IL-2 gene, in lymphocytes? 2) Does oxidative stress induce lipid peroxidation in these cells?

Both questions were answered in studies on human peripheral blood T and B lymphocytes.

Transcription factor DNA-binding studies (Appendix A)

Since this study aims to develop markers of oxidative stress-induced suppression of cellular function, we studied the ability of DNA sequences from the IL-2 promoter to bind to proteins present in nuclei of lymphocytes that are stimulated by mitogens and are commencing proliferation. IL-2 is central to the cellular immune response and inability to express this gene would result in cellular dysfunction of T lymphocytes. B cells were also studied, although they do not express IL-2, because these cells use the same transcription factors for the regulation of other genes. The DNA-binding assay is based on the fact that DNA bound to a protein (the

transcription factor) will move slower during gel electrophoresis and appear higher (closer to the origin) on the gel. The DNA is labeled radioactively, thereby allowing the position of the DNA-protein complex to be determined on the gel. Three DNA-binding activities present in activated lymphocytes were studied: NFkB, AP-1 and NFAT. The cells were subjected to six types and levels of oxidative stress: an enzymatic activity (PAO) generating hydrogen peroxide, irradiation and four concentrations of hydrogen peroxide administered directly to the cells. While un-stimulated T cells do not express NFkB DNA binding, stimulated cells do express this activity and the interaction is specific as shown by its prevention in the presence of a specific competitor. All the types and levels of oxidative stress we employed completely abolished the induction of NFkB DNA-binding in stimulated T lymphocytes.

Protein binding activities to two other DNA sequences (AP-1 and NFAT) were only expressed in stimulated T cells and were abolished by exposing the cells to oxidative stress, similar to the results obtained with the NFkB sequence.

Since three activation-dependent DNA-binding activities (NFkB, AP-1, NFAT) were suppressed by oxidative stress, it was necessary to determine whether oxidative stress affects every DNA-binding activity in the cells in a non-specific manner. To that end, we studied two more DNA-binding activities that are expressed in human T lymphocytes: NRE-A which is expressed constitutively, and AP-3 which was expressed only upon cellular activation. Only the activity of polyamine oxidase suppressed NRE-A DNA-binding while the other types of oxidative stress had no significant effect on the appearance of the NRE-A-protein complex. Only treatment with hydrogen peroxide at the highest concentration (200 μ M) fully abolished both bands of the AP-3 DNA-binding activity.

The effect of oxidative stress on B cell DNA-binding activities was determined, using the three sequences: NFkB, AP-1 and NFAT, which did not bind cognate proteins when nuclear extracts from stressed T cells were studied. The aim was to compare the responses of B and T cells to oxidative stress. Therefore, one mode of oxidative stress (irradiation) that suppressed all three DNA-binding activities in T cell extracts, was employed in these experiments. Irradiation inhibited the expression of all three DNA-binding activities in activated B lymphocytes, similar to the effects of irradiation on T lymphocytes.

Lipid peroxide determination in lymphocytes exposed to oxidative stress (Appendix A)

Since the goal of these studies was to develop markers of oxidative stress in lymphocytes, we measured lipid peroxidation as a biochemical parameter of exposure to oxidants. The basal level of lipid peroxides in T lymphocytes was 2.4 ± 0.7 nmoles lipid hydroperoxides/mg protein. Only treatment with hydrogen peroxide at 20 μ M for 2 hours induced a rise in cellular peroxides to 11.9 ± 1.8 nmoles lipid hydroperoxide/mg protein ($P < 0.0005$), while the other treatments (hydrogen peroxide at 50-200 μ M, polyamine oxidase and irradiation) did not induce any rise in the levels of lipid peroxides above basal levels. Measurements in B lymphocytes did not show enhancement of cellular peroxides by any of the above-mentioned oxidative stresses.

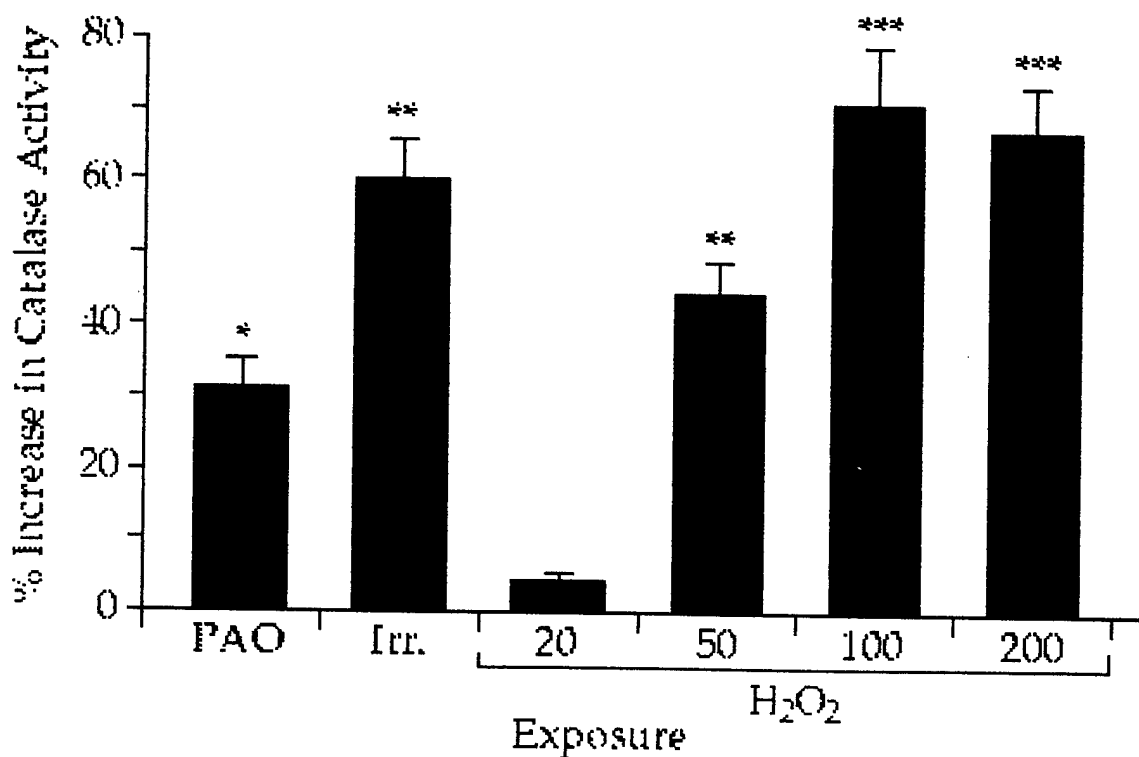
In our Statement Of Work we proposed to answer the following question: To what extent, if at all, are levels of endogenous antioxidant molecules

(catalase, glutathione peroxidase, glutathione, vitamins E and C) altered in human T lymphocytes subjected to oxidative stress?

To answer this question we established in our laboratory the appropriate assays and measured the five parameters in human peripheral blood lymphocytes subjected to different types of oxidative stress.

Catalase activity (Appendix A)

Every oxidative stress exposure except for hydrogen peroxide at 20 μM , induced a significant rise in the cellular catalase activity of T lymphocytes above the basal level (Figure 1). These results suggest that hydrogen peroxide at 20 μM was the only exposure that induced a detectable rise in cellular peroxides (see above) because it was also the only exposure that did not induce the anti-oxidant enzyme catalase. No catalase activity was detected in B lymphocytes probably due to the low numbers of cells available from the peripheral blood.



1. Enhancement of catalase activity by oxidative stress. T cells were exposed to polyamine oxidase (PAO, for 2 days at $5 \times 10^{-4} \text{U/ml}$ + spermidine at $5 \mu\text{M}$, and then washed and incubated for 2 hours in fresh medium), or irradiation (Irr., for 5 minutes at 6 Gy and then incubated for 2 hours in fresh medium), or hydrogen peroxide (H_2O_2 at the indicated concentration, μM , for 2 hours and then washed and incubated for 2 hours in fresh medium). Cells were lysed and cytosolic catalase activity was measured by following kinetically the decomposition of hydrogen peroxide in a spectrophotometer, and calculated per protein concentration. Catalase activity in untreated cells was $1.2 \pm 0.09 \text{ (sec}^{-1} \cdot \text{mg protein}^{-1})$. Results shown are the

increase in catalase activity in stressed cells over untreated cells \pm SD. The increase in catalase activity was significant at $p < 0.01^*$, 0.005^{**} , 0.0005^{***} .

Glutathione peroxidase activity

The basal levels of glutathione peroxidase in T cells were 20.1 ± 2.5 nmoles/mg protein/minute whereas the levels in B cells were approximately 4 times higher - 85.4 ± 9.7 nmoles/mg protein/minute. None of the oxidative stresses affected glutathione peroxidase activities in either the T or the B lymphocytes (Table 1). These results suggest that oxidative stress has a differential effect on catalase (enhancement) versus glutathione peroxidase (no change) activities in exposed lymphocytes.

Table 1

Glutathione peroxidase activities in lymphocytes exposed to oxidative stress

<u>Oxidative stress</u>	<u>T cells</u>	<u>B cells</u>
None	20.1 ± 2.5	85.4 ± 9.7
H ₂ O ₂ 20 μ M	18.9 ± 2.1	87.6 ± 12.0
H ₂ O ₂ 50 μ M	17.9 ± 4.2	79.6 ± 10.4
H ₂ O ₂ 100 μ M	19.4 ± 3.0	88.6 ± 7.9
H ₂ O ₂ 200 μ M	22.8 ± 2.9	75.9 ± 8.4
Irradiation	18.7 ± 2.8	80.4 ± 6.9
Polyamine oxidase	23.8 ± 2.7	89.7 ± 11.8

Lymphocytes were purified, exposed and assayed as mentioned in the Methods section. Glutathione peroxidase activity is expressed as nmoles/mg protein/minute \pm SD, $n=3$.

Reduced glutathione (GSH) levels

A standard of glutathione yielded an excellent calibration curve using the Bioxytech GSH-400 kit (figure 2). However, even when 35 million peripheral blood lymphocytes were used per sample, the basal levels of reduced glutathione were 10.5 ± 0.9 μ M at optical densities of approximately 0.03. Therefore, detecting a decrease in reduced glutathione concentrations upon exposure to oxidative stress became impossible, even when the more sensitive Tietze method was employed.

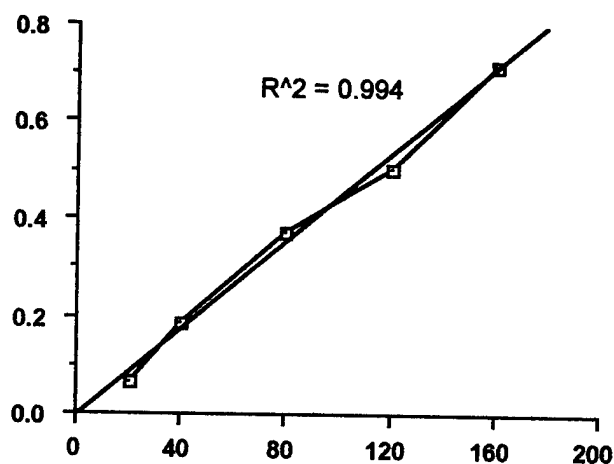


Figure 2.

Vitamin C concentrations

The basal levels of vitamin C in peripheral blood lymphocytes were so low that we had to perform the assay for free in an outside site (Lab Corp., Burlington, NC) that specializes in anti-oxidant measurements, using very sensitive high pressure liquid chromatography methods. Still, 30-50 million cells per sample were required to detect measureable levels of vitamin C. No consumption of vitamin C was noted in cells exposed to various types of oxidative stress (table 2). These results do not support a role for exogenous vitamin C as a protective agent against oxidative stress in human peripheral blood lymphocytes.

Table 2**Vitamin C concentrations in human lymphocytes**

<u>Oxidative stress</u>	<u>Batch 1 (50 million cells/sample)</u>	<u>Batch 2 (35 million cells/sample)</u>
None	0.9	0.4
H ₂ O ₂ 200 μ M	0.8	
Polyamine oxidase		0.5
Irradiation		0.4

T lymphocytes were purified, exposed and assayed as described in the Methods section. Levels of vitamin C are presented as mg/dL.

Vitamin E levels

As for vitamin C, the levels of vitamin E were so low that each sample consisted of approximately 60 million cells. We were able to construct a reliable standard curve (figure 3). Based on this curve we did not detect any depletion of vitamin E from cells exposed to the various types of oxidative stress employed. The basal levels of Vitamin E were $0.6 \pm 0.05 \mu\text{g}/\text{ten million cells}$. These results do not support a role for vitamin E in protecting human peripheral blood lymphocytes against oxidative stress.

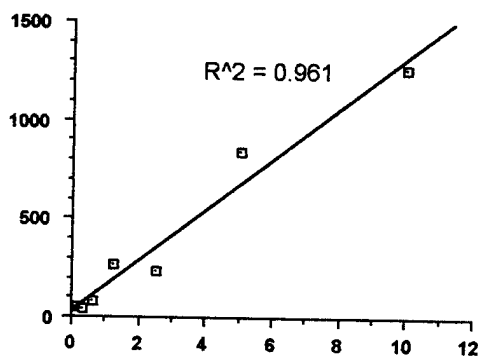


Figure 3.

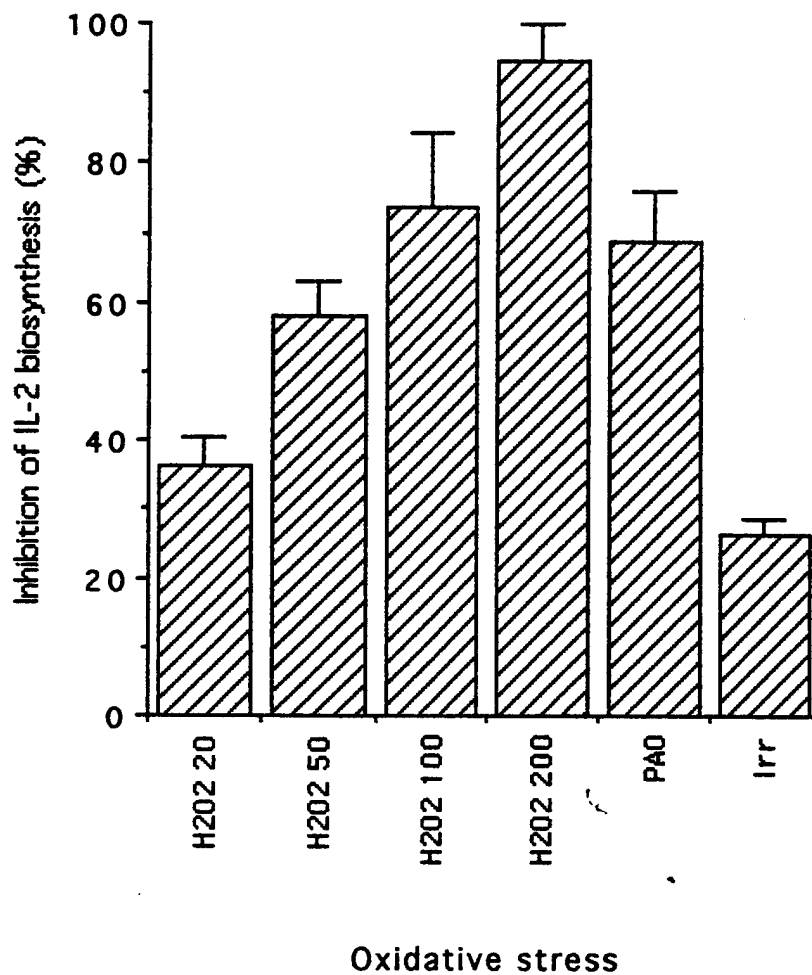
In our Statement of Work we asked the following question: Do pharmacological treatments with antioxidants (N-acetylcysteine=NAC) and/or signal-enhancing drugs (aspirin) protect human T lymphocytes against oxidative stress? Effects on IL-2 biosynthesis were assessed.

Suppression of IL-2 biosynthesis by oxidative stress

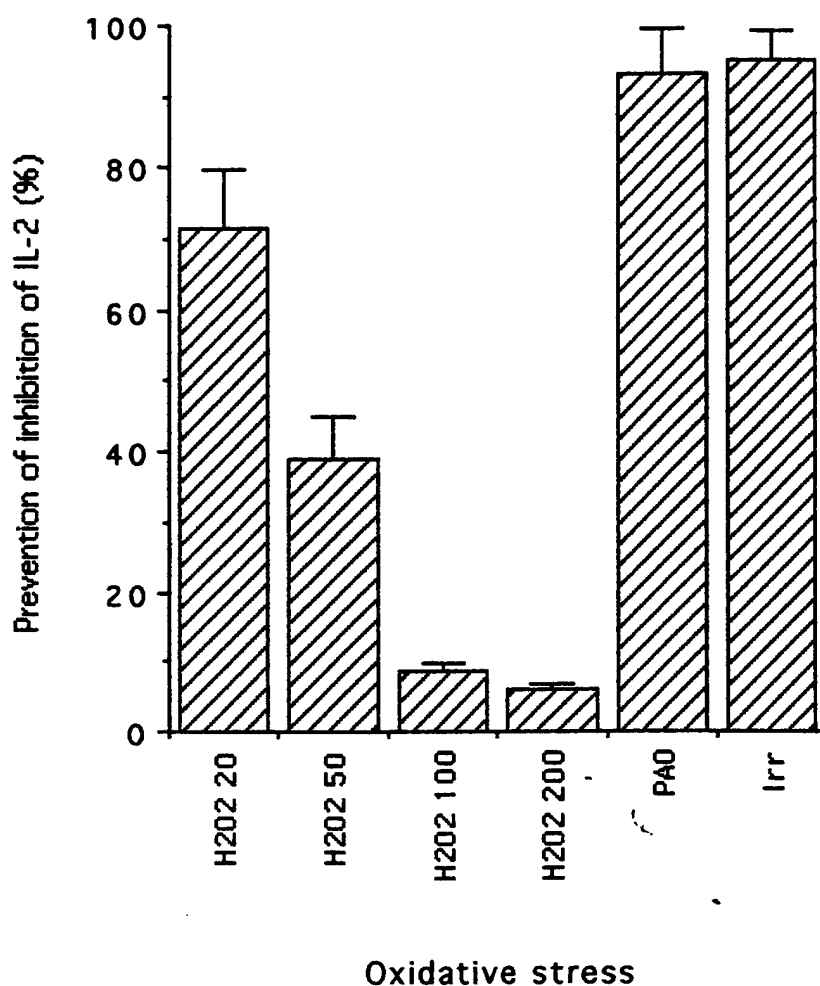
The effects of three different modes of oxidative stress on human T lymphocyte mitogen-induced IL-2 biosynthesis, were compared (Figure 4). Each mode of stress was employed at the highest sub-lethal levels (less than 10% cell death). Namely, H_2O_2 at more than 200 μM , PAO and spermidine at more than 5×10^{-4} u/ml and 5 μM , respectively, and irradiation at more than 6 Gy, caused more than 10% death as assessed by trypan blue exclusion. Acute exposure to H_2O_2 suppressed IL-2 biosynthesis dose-dependently, reaching almost 95% at 200 μM . However, longitudinal exposure to H_2O_2 (at a sub-lethal level) had a similar effect to that of acute H_2O_2 exposure at 100 μM . Interestingly, the highest irradiation dose that caused less than 10% cell death (6 Gy) only suppressed IL-2 biosynthesis by about 26%. Aspirin, although being capable of up-regulating signal transduction in T lymphocytes, did not protect lymphocytes against oxidative stress. We then turned to assess anti-oxidants that modulate intra-cellular glutathione levels.

Prevention of oxidative stress-induced suppression of IL-2 biosynthesis by NAC

NAC, which is both an anti-oxidant and a glutathione precursor, was added to the lymphocytes prior to being exposed to oxidative stress. NAC was used at 3 mM, the highest concentration that was non-toxic for the cells. The ability of NAC to protect against acute exposures to H_2O_2 was inversely proportional to the concentration of H_2O_2 used (Figure 5). At 100 and 200 μM H_2O_2 , NAC only prevented less than 10% of the inhibition of IL-2 biosynthesis, and this effect was not statistically significant. On the other hand, NAC almost abolished completely the suppression of IL-2 biosynthesis caused by longitudinal exposure to H_2O_2 and irradiation (Figure 5).



4. Suppression of mitogen-induced IL-2 biosynthesis by oxidative stress. T cells were pre-treated with polyamine oxidase (PAO, for 2 days at 5×10^{-4} u/ml + spermidine at $5 \mu\text{M}$, and then washed and incubated for 2 hours in fresh medium), or irradiation (Irr, for 5 minutes at 6 Gy and then incubated for 2 hours in fresh medium), or hydrogen peroxide (H_2O_2 , for 2 hours at the indicated concentration, μM , and then washed and incubated for 2 hours in fresh medium). Cells were then stimulated with PHA ($1 \mu\text{g}/\text{ml}$) + TPA ($5 \text{ ng}/\text{ml}$) for 24 hours. The supernatants of the cell cultures were collected and IL-2 concentration was determined by ELISA. The IL-2 concentration generated by control cultures that were not exposed to oxidative stress prior to mitogenic stimulation, defined as 100%, was $394.2 \pm 37.8 \text{ pg}/\text{ml}$. $P < 0.0005$ was calculated for the difference between IL-2 production in each oxidative stress-exposed culture and the production in control cultures. Percentages of inhibition are presented \pm SD, $n=5$.



5. Protection by NAC against oxidative stress-induced suppression of IL-2 biosynthesis. T cells were incubated with or without 3 mM NAC for 3 days. Cells were washed and NAC was replenished daily because of the lability of NAC. Cells were then washed, exposed to oxidative stresses and stimulated as described in Figure 4. IL-2 concentrations in culture supernatants were determined by ELISA. Values presented are the percentages \pm SD, by which NAC decreased the inhibition of IL-2 biosynthesis in cultures pre-incubated with NAC, in comparison to cultures that were exposed to the same oxidative stress but were not pre-incubated with NAC, $n=5$. NAC decreased the suppression of IL-2 biosynthesis, $p<0.0005$, at all oxidative stress conditions except for H_2O_2 at 100 and 200 μM where NAC had a statistically non-significant effect.

Studies benefiting from the infra-structure supported by this grant (Appendices B, C)

Up to here, we described the results of research that was directly funded by this grant. The infrastructure created in our laboratory (including the purification of blood cells, maintaining of an epithelial cell line and establishment of the electrophoretic mobility shift assay) allowed the graduate students of the principal investigator to perform their separate research activities. Although these activities were in no way directly supported by this grant, it is appropriate to recognize the fact that the students' research (which is ancillary to the grant) benefited indirectly from the grant. Describing these results is further justified by the general areas of the students' projects: the signal transduction cascade initiated in lung cells by the oxidant ozone; and cellular stress in transformed lymphocytes.

Ozone is a major environmental oxidant which is generated by UV radiation and high-voltage sparks. The oxygen radicals it generates are similar to those encountered by tissues during reperfusion. We found that the lung inflammation induced by this oxidant is mediated through the secretion of the neutrophil chemotactic factor - IL-8. The lung epithelial cells respond directly to ozone. Consequently, a signaling cascade is initiated in which oxidative stress and protein tyrosine kinases, as well as cAMP-dependent kinases, transmit the signal from the plasma membrane to the nucleus. There, several transcription factors including NF κ B, NF-IL-6 and AP-1, enhance the expression of a set of genes including IL-8. The ensuing biosynthesis and secretion of this cytokine results in neutrophil recruitment to the alveolar lumen and in other characteristics of lung inflammation. Identification of the cellular events necessary for ozone-induced lung inflammation to occur, may allow the development of means to control and suppress this histopathological response.

Apoptosis is a form of active cell death which occurs in response to severe cellular stresses including oxidative stress. There are instances where clinicians are interested in inducing this "suicide" path in cells. A prominent example is the death of cancer cells. Many anti-cancer drugs kill their target cells by inducing apoptosis. Unfortunately, cancer cells have developed an array of mechanisms to avoid death, including the activity of an efflux pump (P-glycoprotein=P-gp) that removes anti-cancer drugs from intra-cellular compartments. To counteract this defensive response, inhibitors of P-gp have been found that enhance the effectiveness of anti-cancer drugs. We studied the ability of three P-gp inhibitors (verapamil, MRK16-a monoclonal antibody against P-gp, and PSC 833-a non-immunosuppressive derivative of cyclosporin D) to kill human T lymphoma cells directly, by inducing apoptosis. Based on morphological and biochemical criteria, P-gp inhibitors induced apoptosis in transformed T lymphocytes but not in their normal peripheral blood counterparts. PSC 833 At the same concentrations is currently used in advanced clinical trials as a modulator of drug resistance. Since anti-cancer drugs were not present in our experimental cultures we conclude that transformed T cells require P-gp to maintain viability, and that the enhancement of anti-cancer drugs efficacy by P-gp inhibitors may be partially explained by the ability of the latter to directly kill lymphoma cells.

The transcription factor NF-IL-6 enhances the expression of the gene (MDR1) that encodes P-gp. Since the expression of NF-IL-6 is regulated by protein kinase C (PKC), and we formerly found that non-steroidal anti-inflammatory drugs (NSAID) induce PKC activity in human T lymphocytes, we assessed the ability of NSAID to enhance P-gp expression and function. Aspirin and sodium salicylate, at plasma

attainable levels, induced the expression of NF-IL-6 DNA-binding, P-gp mRNA, protein and function, in a human T lymphoma cell line. These results raise the possibility that the use of NSAID may be contra-indicated during cancer chemotherapy sessions.

Discussion

Three modes of oxidative stress: hydrogen peroxide, polyamine oxidase activity generating hydrogen peroxide gradually, and irradiation, suppressed the activation-dependent DNA-binding activities of three transcription factors: NF κ B, AP-1 and NFAT, in human peripheral blood T lymphocytes. Only exposure to polyamine oxidase and hydrogen peroxide at 200 μ M suppressed the DNA-binding activities of NRE-A and AP-3, respectively. Similar to T cells, the DNA-binding activities of NF κ B, AP-1 and NFAT were also suppressed by irradiation in human peripheral blood B lymphocytes. Only exposure to hydrogen peroxide at 20 μ M generated measurable lipid peroxidation products in T lymphocytes.

We have previously reported that exposure to polyamine oxidase results in suppression of transmembrane signal transduction in human peripheral blood T lymphocytes. This leads to suppression of the activation-dependent expression of transcription factors in the nucleus, and finally to inhibition of the transcription of the IL-2 gene. In the current study, we compared the effects of different types and levels of oxidative stress on nuclear signal transduction in exposed human lymphocytes. Three transcription factor DNA-binding activities were suppressed in T lymphocytes by every condition of oxidative stress employed, while two transcription factors were mostly unmodulated. Our results suggest that activation-induced DNA-binding activities of NF κ B, AP-1 and NFAT may serve as sensitive markers of oxidative stress in human peripheral blood T lymphocytes. Moreover, these activities respond to either acute or chronic stresses involving hydrogen peroxide as well as hydroxyl radicals. The restricted response of NRE-A DNA-binding to low and chronic stress (polyamine oxidase activity), and the restricted response of AP-3 DNA-binding to high and acute stress (hydrogen peroxide at 200 μ M), may allow the use of these activities as markers for the respective specific modes of oxidative stress. The reasons for these restricted responses are unclear and may be related to the signaling pathways leading to the nuclear expression of NRE-A and AP-3.

The DNA-binding activities of NF κ B and AP-1 are induced upon exposure to oxidants. The apparent contradiction with our results may be resolved by recognizing that in our system (but not in other studies), cells were incubated for 2 hours in fresh medium after the exposures, followed by stimulation for 6 hours, and only then were DNA-binding activities determined. Therefore, we are assessing the effects of oxidative stress on T and B cell mitogenic activation rather than the direct effect on transcription factor activities. In this context, the decline in IL-2 production by human T lymphocytes from aged persons is associated with impaired activation of AP-1 and NFAT. In view of the oxygen radical-related theory of aging, this is potentially an example of T cell suppression at the transcription factor level by oxidative stress in vivo.

We found that radiation suppressed DNA-binding activities in activated T and B lymphocytes. Our results suggest DNA-binding activities as measures of functional suppression of lymphocyte activation by radiation.

Since NF κ B and AP-1 are the most important IL-2 cis-regulatory elements in normal T cells, our results strongly suggest that suppression of transcription factor

function contributes to the down-regulation of IL-2 production and of cellular activation caused by inducing oxidative stress in human lymphocytes.

Direct measurements of lipid hydroperoxides did not detect increased levels following oxidative stress, except for exposure of T lymphocytes to the lowest concentration of hydrogen peroxide. A possible explanation to these findings is that the direct biochemical damage was repaired within two hours after the exposures. The lowest concentration of hydrogen peroxide may not have been sufficient to induce appropriate levels of anti-oxidant defenses, allowing the lipid peroxidation to be detected. On the other hand, all 3 modes of oxidative stress resulted in suppression of cellular function that was clearly evident even 8-26 hours after the exposures, as judged by transcription factor activities. We have previously found that oxidative stress suppresses early signal transduction steps, protein tyrosine phosphorylation and calcium mobilization. Therefore, the eventual suppression of transcription factor activities may actually reflect early effects of oxidative stress on lymphocyte transmembrane signal transduction. These effects are apparently not reversible, at least within 2 hours after the stress.

Three modes of oxidative stress: hydrogen peroxide, polyamine oxidase activity generating hydrogen peroxide gradually, and irradiation, enhanced catalase activity in human peripheral blood T lymphocytes. Glutathione peroxidase activity and reduced glutathione, vitamin C and vitamin E levels, were not modulated by oxidative stress. Only exposure to hydrogen peroxide at 20 μ M did not enhance catalase activity in T lymphocytes.

As mentioned above, direct measurements of lipid hydroperoxides did not detect increased levels following oxidative stress, except for exposure of T lymphocytes to the lowest concentration of hydrogen peroxide (20 μ M). We proposed a possible explanation to these findings suggesting that the direct biochemical damage by this low stress was not enough to induce endogenous anti-oxidant mechanisms and therefore allowed for the detection of lipid peroxidation. Our current results strongly support our proposal, i.e., catalase activity was enhanced by each type of oxidative stress except for the weakest stress which in turn did cause detectable lipid peroxidation. Therefore, lipid peroxidation appears to be prevented, or at least reversed, by catalase activity. Interestingly, glutathione peroxidase which is another major hydrogen peroxide scavenger did not show enhanced activity upon exposure to oxidative stress. The reasons for this are unclear and may be related to the specific biology of peripheral blood lymphocytes.

We assessed the modulation of the endogenous levels of two anti-oxidants (vitamins C and E) by oxidative stress. It was assumed that if these anti-oxidants play an important role in the defense of the cells against oxidative stress, their levels should decrease upon exposure to oxidants. This did not turn out to be the case. Again, it seems that catalase is the most important endogenous anti-oxidant agent from among the four we measured. The fifth, reduced glutathione, occurred at levels that were practically below our detection ability in peripheral blood lymphocytes.

Comparing three modes and different intensities of oxidative stress, we found that each of the stresses suppressed mitogen-induced IL-2 biosynthesis by human peripheral blood T lymphocytes. The extent of suppression differed between the various stresses employed. NAC prevented oxidative stress-induced suppression of IL-2 biosynthesis, except for that induced by the highest levels of acute stress.

Acute stress induced by reagent hydrogen peroxide suppressed IL-2 biosynthesis in a dose-dependent fashion, reaching almost complete abolishment at the highest concentration studied. As each stress was employed at the highest possible

sub-lethal levels, the results suggest that cells exposed to longitudinal stress and irradiation can not exhibit complete suppression of IL-2 biosynthesis, probably because intensities high enough to achieve such response would be lethal.

NAC proved to be limited in its ability to protect human T lymphocytes against acute oxidative stress-mediated suppression of mitogen-induced IL-2 biosynthesis, being practically useless at 100-200 μ M of hydrogen peroxide. On the other hand, it was very efficient in preventing longitudinal (mediated by PAO) and irradiation-induced stresses. Since these are environmentally-encountered stresses, NAC appears to be a promising candidate for a molecule capable of providing defense to individuals exposed to conditions which generate reactive oxygen intermediates. A similar level of protection by NAC against acute stress was only evident at 20 μ M hydrogen peroxide. At this level, NAC brought the level of IL-2 biosynthesis to almost 90% of the un-suppressed cells control level, due to the combination of low suppression and high prevention by NAC. Interestingly, this is the only type of stress where we reported the detectability of lipid peroxides, probably due to lack of induction of catalase (Appendix A). These findings suggest that exogenous NAC can protect cells which are exposed to low-level acute stress and do not express the endogenous anti-oxidant molecule – catalase. Notably, NAC was used in these experiments at its highest non-toxic level (3 mM).

In accordance with our results showing protection by NAC against oxidative stress-induced suppression of IL-2 biosynthesis, this low-molecular-weight anti-oxidant increased glutathione levels and enhanced the anti-tumor responses of lymphokine-activated killer cell. Furthermore, NAC administered in vivo prevented changes in the levels of plasma lipid peroxides and reduced the pathophysiological consequences of aortic clamping-induced ischemia-reperfusion syndrome. Another area where oxidative stress appears to have pathological significance is that of rheumatic diseases. We have shown the involvement of reactive oxygen intermediates in the suppression of IL-2 biosynthesis exhibited by lymphocytes from rheumatoid arthritis patients. NAC suppressed type II collagen-induced arthritis in mice, probably by controlling the oxidative stress-derived damage associated with this disease.

One of the main goals of this work was to identify markers of exposure to oxidative stress. As we reported, lipid peroxidation can be detected only in cells exposed to weak levels of stress, while the DNA-binding activities of IL-2 expression-regulating transcription factors are suppressed by every stress studied and the assay is not quantitative. However, measurements of IL-2 levels by ELISA appear to provide a much better parameter of oxidative stress-induced suppression of human lymphocyte function. It is a quantitative parameter which responds dose-dependently to acute stress, and is also suppressed significantly by longitudinal stress and irradiation.

Occupational and environmental settings include the exposure of individuals to smoke, toxic chemicals and combustion products which contain (or may generate) very high concentrations of oxidants including peroxides and peroxy radicals. We used ex vivo peripheral blood cells which are a physiological target of oxidants and therefore are an appropriate model to identify toxic hazards, and a system that allows follow-up of exposed individuals. Our results provide the scientific basis for a quick, inexpensive, in vitro test method (IL-2 ELISA) to identify immunotoxic oxidative hazards and create a health hazard data base of such compounds. This method may also contribute to the identification of individuals exposed and affected by oxidants, by testing their blood. Finally, through the use of this method it will be possible to

monitor the success of anti-oxidant prevention/treatment schemes (e.g., by NAC) in humans exposed to oxidants.

We hypothesized that: 1) Oxidative stress alters the expression of transcription factors in human T lymphocytes; and, 2) The effects of oxidative stress on human lymphocytes are modulated by endogenous mechanisms and can be reversed by exogenous therapeutic measures. These hypotheses were proven correct. Different oxidative stress modes suppressed mitogen-induced transcription factor activities, and appear to be modulated by endogenous catalase activity, as well as exogenously added N-acetylcysteine.

Conclusion

We proved our hypothesis that oxidative stress alters the expression of transcription factors in human lymphocytes. Oxidative stress suppressed the activation-dependent DNA-binding activities of three transcription factors: NF κ B, AP-1 and NFAT, in human peripheral blood T lymphocytes. Similar to T cells, the DNA-binding activities of NF κ B, AP-1 and NFAT were also suppressed by irradiation in human peripheral blood B lymphocytes. These results suggest that transcription factor functions can be used as markers of blood lymphocyte exposure to oxidants. Several modes of oxidative stress were studied, including low level longitudinal stress mimicking exposures to environmental chemical toxicants. Since the transcription factors under study are essential for optimal expression of IL-2, and therefore indirectly also for successful activation and function of the cellular immune response, suppression of these nuclear signaling events reflects not only exposure but also the detrimental effect of exposure to oxidative stress inducers. These studies were conducted with human peripheral blood lymphocytes which are readily available, and should therefore be amenable to development into population-based markers of environmental exposure to oxidants.

Only exposure to hydrogen peroxide at 20 μ M generated measurable lipid peroxidation products in T lymphocytes while lipid peroxidation was undetectable in B lymphocytes exposed to any of the modes of oxidative stress under study. These were unexpected results since we assumed that lipid peroxidation, being a biochemical hallmark of cellular oxidative stress, will occur upon exposure of lymphocytes to oxidants. A possible explanation may be that anti-oxidant defenses are mounted by stressed lymphocytes, which repair the structural damage. On the other hand, the damage to signal transduction in activated lymphocytes is sufficient to persist in spite of the cellular anti-oxidative defenses. Therefore, our results suggest that a functional parameter (nuclear signal transduction) is much more sensitive than a structural parameter (lipid peroxidation) as a marker of oxidative damage to human blood lymphocytes.

Military occupational settings include the exposure of soldiers to smoke and combustion products which contain very high concentrations of oxidants including peroxides and peroxy radicals. Also, oxidative damage may occur secondary to biological or chemical weapon exposure. Serious trauma is another instance where the resultant ischaemia and reperfusion can cause oxidative stress and tissue antioxidant depletion.

The vast majority of studies into human T cell signal transduction and gene regulation were carried out using transformed cell lines. We, however, use ex vivo peripheral blood cells which are a physiological target of oxidants and therefore are

the most appropriate model to identify toxic hazards, and the only system that allows follow-up of exposed individuals.

The results and conclusions of this project's first year provide three direct benefits:

1. The scientific basis for a quick, inexpensive, in vitro test method (electrophoretic mobility shift assay to determine transcription factor binding to cognate DNA sequences) to identify immunotoxic oxidative hazards and create a health hazard data base of such compounds.
2. This method will allow the identification of personnel exposed and affected by oxidants, by testing their blood.
3. Through the use of this method it will be possible to monitor the success of anti-oxidant prevention/treatment schemes in soldiers exposed to oxidants, or in wounded soldiers suffering of reperfusion-associated oxidative stress.

Assessing the effects of oxidative stress on endogenous anti-oxidant molecules revealed dramatic differences between different anti-oxidants. It seems that only catalase activity is modulated by oxidative stress, putatively being recruited to protect against the stress. On the other hand, two classical anti-oxidants (vitamins C and E) are not consumed during the stress period. This questions the importance of these agents as supplements aimed at providing protection against oxidative stress in lymphocytes.

The results of this project's second year provide the following two conclusions:

1. Enhanced catalase activity may be developed as a marker for the ability of cells from exposed soldiers to mount an anti-oxidative response.
2. Vitamins C and E were apparently not part of the anti-oxidative defense arsenal of the lymphocyte

The results of this projects' third year provide the following conclusions:

1. NAC appears to be a promising candidate for a molecule capable of providing defense to individuals exposed to conditions which generate reactive oxygen intermediates.
2. Exogenous NAC can protect cells which are exposed to low-level acute stress and do not express the endogenous anti-oxidant molecule – catalase.
3. IL-2 biosynthesis (measured by ELISA) can serve as an indicator of exposure to environmental oxidants.

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Oxidative stress suppresses transcription factor activities in stimulated lymphocytes

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SUMMARY

Effects of oxidative stress on stimulation-dependent signal transduction, leading to IL-2 expression, were studied. Purified quiescent human blood T lymphocytes were subjected to: (i) acute exposure to hydrogen peroxide; (ii) chronic exposure to hydrogen peroxide; and (iii) acute exposure to ionizing radiation. The cells were then stimulated for 6 h. DNA-binding activities (determined by the electrophoretic mobility shift assay) of three transcription factors: NF κ B, AP-1 and NFAT, were abolished in the lymphocytes by all three modes of oxidative stress. The lymphocytes exhibited lipid peroxidation only upon exposure to the lowest level of hydrogen peroxide used (20 μ M). All three modes of oxidative stress induced catalase activity in the lymphocytes. The only exception was hydrogen peroxide at 20 μ M, which did not induce catalase activity. We conclude that: (i) suppression of specific transcription factor functions can potentially serve as a marker of exposure to oxidative stress and its effects on human lymphocytes; (ii) lipid peroxidation is only detectable in human lymphocytes upon exposure to weak oxidative stress which does not induce catalase activity; (iii) therefore, transcription factor DNA-binding activities are more sensitive to oxidative stress than lipid peroxidation.

Keywords oxidative stress immunosuppression NF κ B NFAT AP-1

INTRODUCTION

T lymphocytes are activated following the binding of a ligand to the antigen receptor complex. One of the early manifestations of this interaction is the transcriptional activation of the IL-2 gene [1]. IL-2 is a pivotal lymphokine involved in B and T lymphocyte, as well as natural killer (NK) cell regulation [1]. The modulation of IL-2 transcription by nuclear proteins can serve as a general readout that would be affected by any abnormality occurring earlier in the activation pathway. A transcriptional enhancer in the promoter region of the IL-2 gene responds to signals generated after activation through the T cell antigen receptor [2]. A number of positive regulatory elements have been identified in this region, including: NFAT, AP-1, NF κ B, EGR-1, AP-3, Oct-1, and Sp1 [3-5]. Transcription factors binding to the first three, which are expressed in primary human T cells only upon stimulation, are plausible targets for suppression of T cell activation.

A variety of distinct biochemical changes in lymphocytes and in various other target cells is induced by the oxidants hydrogen peroxide and hydroxyl radical. These changes include alterations in enzymatic activities, lipid peroxidation and damage to DNA. Ionizing radiation can be used as a means of introducing oxygenating

radicals into lymphocytes in a geometrically and temporally precise way. The absorption of radiation involves splitting H₂O molecules (the most common constituent of cells) into hydroxyl radicals and H \bullet radicals which are initially distributed in proportion to the radiation dose distribution [6]. In addition, irradiation of dissolved O₂ will produce the superoxide radical, HO₂ \bullet , also following the radiation dose distribution. The superoxide radical has intermediate reactivity between that of strongly reactive hydroxyl radicals and relatively weakly reactive hydrogen peroxide.

We have previously described a new mechanism of IL-2 down-regulation [7]. Endogenous hydrogen peroxide produced by monocytes and endogenously produced or exogenously added polyamines provide down-regulatory signals for IL-2 production by human peripheral blood T cells. The interaction between polyamine oxidase (PAO; EC 1.4.3.4, monoamine oxidase) and the polyamine spermidine generates products (including hydrogen peroxide) over 2 days that provide chronic low-level oxidative stress, suppressing IL-2 production. Furthermore, we found that PAO activity suppressed protein tyrosine phosphorylation, calcium mobilization and nuclear DNA-binding activities [8].

The objective of this study was to compare the effects of three modes of inducing oxidative stress in human lymphocytes on stimulation-induced transcription factors, in order to facilitate the development of functional markers for the exposure to, and effect of, oxidative stress in man. Oxidative stress was exerted on unstimulated

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cells, since the vast majority of peripheral blood lymphocytes (PBL) are in a quiescent state. The modes studied were: (i) high levels of reagent hydrogen peroxide generating short but acute stress; (ii) PAO activity generating extracellularly low levels of hydrogen peroxide for 2 days; and (iii) electron irradiation generating both extra- and intracellularly mainly hydroxyl radicals.

MATERIALS AND METHODS

Cells

T cells from the peripheral blood of healthy donors were studied. Cells were incubated for all assays in a serum-free medium, because fetal calf serum (FCS) contains PAO activity [7]. Therefore, RPMI 1640 with Nutridoma-HU supplement (Boehringer Mannheim Biochemicals, Indianapolis, IN) was used.

Lymphocyte preparation. Cells were purified by Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation. The resultant mononuclear cell preparation was allowed to adhere to plastic dishes to remove macrophages and other adherent cells.

Non-adherent mononuclear cells were mixed with a suspension of neuroaminidase-treated sheep erythrocytes and incubated at 37°C for 15 min, followed by centrifugation and further incubation at 4°C for 45 min. Thereafter, the rosetted cells were obtained by centrifugation through Ficoll-Hypaque. The erythrocytes in the cell pellet were lysed by exposure to 0.83% NH_4Cl . The rosetted cells contained >98% $\text{CD}3^+$ T cells, and 0.4–1% $\text{M}3^+$ monocytes as determined by flow cytometry.

Oxidative stress

These modes were used: (i) a short high-level extracellular stress—reagent hydrogen peroxide was added directly at 20, 50, 100 and 200 μM for 2 h. We have found that these levels suppress IL-2 production in human blood lymphocytes without affecting cell viability [9]; (ii) a longitudinal low level extracellular stress—lymphocytes were preincubated for 2 days with a commercial preparation of PAO (a monoamine oxidase which oxidizes polyamines at a 2.5-fold higher rate than benzylamine [7]; Sigma, St Louis, MO) at 5×10^{-4} U/ml and spermidine at 5 μM . This exposure generates gradually 5 μM hydrogen peroxide over 2 days and suppresses IL-2 production in response to mitogenic stimulation [7]; (iii) electron radiation generating both extra- and intracellularly mainly hydroxyl radicals—lymphocytes were exposed to a radiation dose of 6 Gy for 5 min. This dose produces non-lethal cellular responses [10] and generates oxidants per time unit at about 20-fold higher levels than mode (ii), but for a much shorter period of time. We used a 2.5-MeV Van de Graaff accelerator that generated electrons to a maximum energy of 1.8 MeV. The cells were exposed in suspension to high energy x-rays generated by stopping the electron beam in a tantalum plate. Doses were continuously monitored by means of parallel plate ionization chambers coupled with a stable, vibrating reed electrometer.

Oxidative stress was exerted and the cells were washed and rested for 2 h before stimulation in order to exclude any possible effects on the assay used.

Measurements of transcription factor activities

For T cell stimulation we used phytohaemagglutinin (PHA; 1 $\mu\text{g}/\text{ml}$) + tetradecanoyl phorbol acetate (TPA; 5 ng/ml), for 6 h at 37°C, 5% CO_2 , before collecting the cells for nuclear extraction.

DNA-binding determination by the electrophoretic mobility shift assay

Preparation of nuclear extracts. Cells were washed and nuclear extracts were prepared according to a modification of the method of Schreiber *et al.* [11]. This method is suitable for small numbers of cells and therefore appropriate (based on our experience; [8,12]) for studies of PBL. Cells were washed and resuspended in Tris-buffered saline, transferred to an Eppendorf tube and pelleted. The cell pellet was resuspended in a buffer containing 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 5 $\mu\text{g}/\text{ml}$ aprotinin, 5 $\mu\text{g}/\text{ml}$ antipain, 100 μM benzamide, 5 $\mu\text{g}/\text{ml}$ leupeptin, 5 $\mu\text{g}/\text{ml}$ pepstatin, 5 $\mu\text{g}/\text{ml}$ soybean trypsin chymotrypsin inhibitor, pH 7.9. The cells were allowed to swell on ice for 15 min and NP-40 at 0.625% was added. The tube was vortexed for 10 s and centrifuged for 30 s in a microfuge. The nuclear pellet was resuspended in a buffer containing 20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and the seven aforementioned protease inhibitors, pH 7.9. The tube was vigorously rocked on wet ice for 15 min on a shaking platform, and the nuclear extract was centrifuged for 5 min to remove insoluble nuclear matrix. The protein concentration of the supernatant was determined (Bradford method; BioRad Protein Assay Kit (Hercules, CA)). Aliquots were stored at -70°C .

DNA-protein interactions. DNA probes containing the binding sites from the IL-2 promoter region [3] were purchased from Genosys (The Woodlands, TX). The probe for NFAT-1 spans between nucleotides -255 and -285: 5'-GGAGGAAAACTGTTT-CATACAGAAGGCGTT-3'. The probe for AP-1 spans between nucleotides -140 and -156: 5'-TTCAAAGAGTCATCAG-3'. The probe for NF κ B spans between nucleotides -190 and -214: 5'-TAACAAACAGGGATTTCACCTACAT-3'.

The probes were labelled with ^{32}P -ATP using T4 polynucleotide kinase (Promega, Madison, WI). For the binding assay, 10 000 cpm DNA probe (≈ 0.2 ng) were combined with 2 μg poly (dI-dC) (a non-specific competitor DNA), 3 μg bovine serum albumin (BSA; a protein carrier) and 10 μg nuclear extract in a final reaction volume of 20 μl . The binding reaction mixture was incubated for 15 min in a 30°C water bath. The protein-DNA complexes were detected on a 4% low ionic strength native polyacrylamide gel. The gel was dried under vacuum and autoradiographed.

Measurements of lipid peroxidation

Quantitative peroxide assay. A lipid-compatible formulation of the PeroXOquant Quantitative Peroxide Assay (Pierce Chemical Co., Rockford, IL) was used. This assay is adapted to measure cellular hydroperoxides. To differentiate between hydrogen peroxide and peroxides of cellular molecules (such as lipid peroxides) we followed the recommendations of the manufacturer and regarded any catalase (7000 U/ml)-inhibitable measurement as representing hydrogen peroxide. In the assay, peroxides convert Fe^{2+} to Fe^{3+} in a sulphuric acid solution. The Fe^{3+} complexes with the xylenol orange dye to yield a purple product with absorbance at 540–600 nm. The molar extinction coefficient of the xylenol orange- Fe^{3+} complex is $1.5 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ in 25 mM H_2SO_4 at room temperature. Five million cells were lysed by sonication (two 10-s pulses with a 10-s interval) and incubated for 15–20 min at room temperature in the following working solution (10 times the volume of the sonicate): 0.25 mM ammonium ferrous (II) sulphate, 25 mM H_2SO_4 , 4 mM butylated hydroxytoluene (BHT), 125 μM xylenol orange in methanol. Results were read at 595 nm in a microtitre plate reader. For calibration and validation, a series of

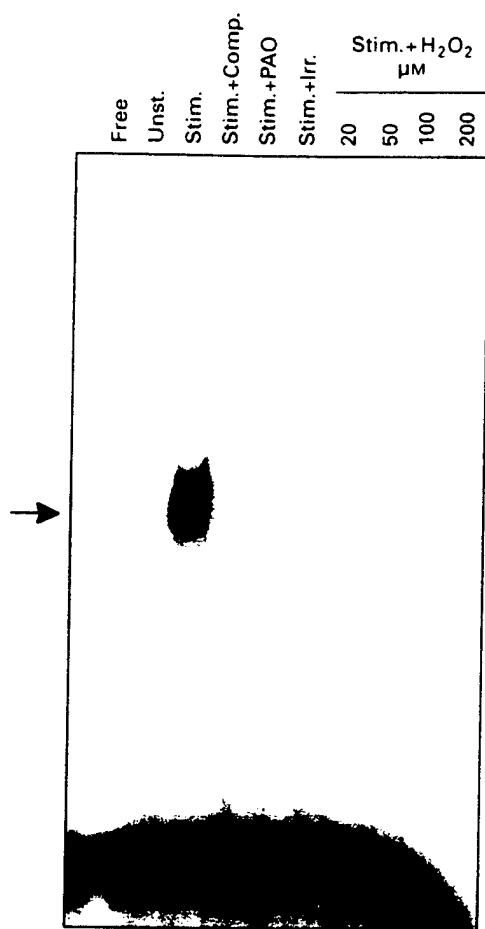


Fig. 1. Suppression of NFκB DNA-binding by oxidative stress. T cells were pretreated with polyamine oxidase (Stim. + PAO, for 2 days at 5×10^{-4} U/ml + spermidine at $5 \mu\text{M}$, and then washed and incubated for 2 h in fresh medium), or irradiation (Stim. + Irr., for 5 min at 6 Gy and then incubated for 2 h in fresh medium), or hydrogen peroxide (Stim. + H_2O_2 , for 2 h at the indicated concentration, and then washed and incubated for 2 h in fresh medium). Cells were then stimulated with phytohaemagglutinin (PHA; $1 \mu\text{g}/\text{ml}$) + tetradecanoyl phorbol acetate (TPA; $5 \text{ ng}/\text{ml}$) for 6 h. In addition, control cultures of untreated cells were either not stimulated (Unst.) or stimulated with PHA + TPA for 6 h (Stim.). Nuclear extracts were prepared and $10 \mu\text{g}$ of protein were incubated with ^{32}P -labelled NFκB sequence and electrophoresed. The lanes were loaded with DNA without nuclear extract (Free), DNA with extract from untreated and unstimulated cells (Unst.), DNA with extract from untreated and stimulated cells (Stim.), same as Stim. + $50\times$ excess of unlabelled probe (Stim. + Comp.), and DNA with extracts from pretreated cells that were also stimulated (Stim. + PAO, Stim. + Irr., Stim. + H_2O_2). The gel was dried and autoradiographed. The arrow marks the specific DNA-protein complex.

hydrogen peroxide solutions at concentrations between $1 \mu\text{M}$ and 1 mM were prepared and assayed. Results were calculated per protein concentration as determined by the Bradford method. Since the peroxide assay allows measurement of peroxides without lipid extraction, a blank without ammonium ferrous (II) sulphate and H_2SO_4 was used to subtract endogenous iron (and other transition metal) readings [13].

Measurements of catalase activity

Ten million cells were lysed by sonication (two 10-s pulses with a 10-s interval) in 0.5 ml PBS. The resultant sonicate was centrifuged

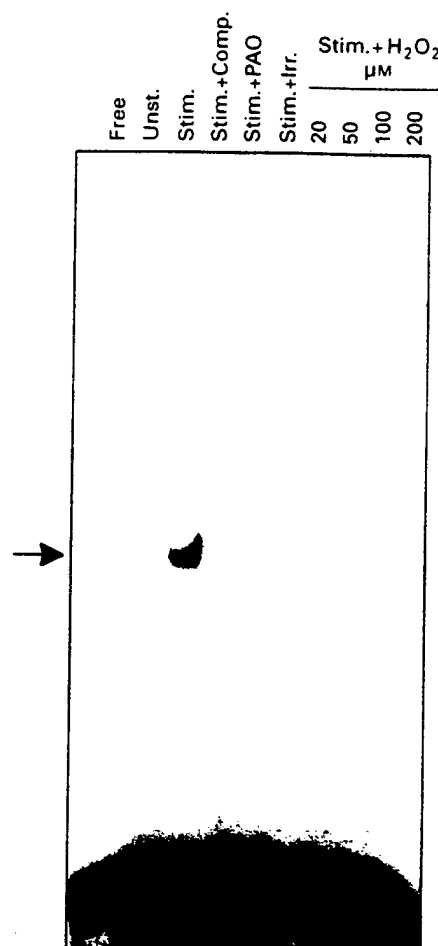


Fig. 2. Suppression of AP-1 DNA-binding by oxidative stress. Same as Fig. 1, except that ^{32}P -labelled AP-1 sequence was used.

at $14\,000g$ for 10 min at 4°C . Catalase activity was measured in the supernatant. Supernatant ($50 \mu\text{l}$) was mixed with $600 \mu\text{l}$ of 15 mM H_2O_2 in a cuvette. The kinetics of the decrease in light absorbance at 240 nm (H_2O_2 decomposition) were determined for 3 min in a DU 640 spectrophotometer (Beckman, Fullerton, CA). A cuvette containing only PBS served as blank. A cuvette without a sample was used to ensure that H_2O_2 did not decompose spontaneously under our experimental conditions. Enzymatic activity was expressed as the rate constant of a first-order reaction (k) divided by the protein concentration. A_1 and A_2 refer to the absorbance before and after a given time interval of measurement (t), respectively. $k = (2.3/t) (\log A_1/A_2) (\text{s}^{-1}, \text{mg protein}^{-1})$ [14,15].

Reagents

All reagents were purchased from Sigma, unless otherwise stated.

Statistical analysis

Data were analysed, where appropriate, using Student's *t*-test.

RESULTS

Transcription factor DNA-binding studies

Since this study aimed to develop markers of oxidative stress-induced suppression of cellular function, we studied the ability of DNA sequences from the IL-2 promoter to bind to proteins present in nuclei of lymphocytes that are stimulated by mitogens and are

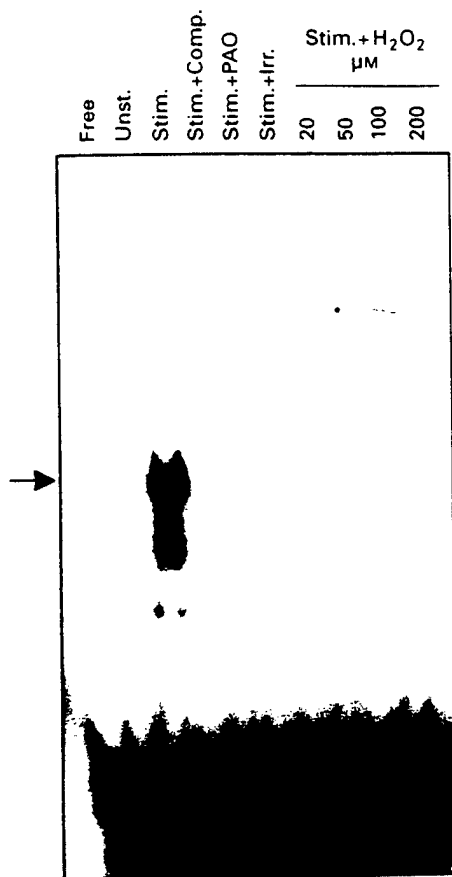


Fig. 3. Suppression of NFAT DNA-binding by oxidative stress. Same as Fig. 1, except that 32 P-labelled NFAT sequence was used.

commencing proliferation. IL-2 is central to the cellular immune response, and inability to express this gene would result in cellular dysfunction of T lymphocytes. Three DNA-binding activities present in activated lymphocytes were studied: NF κ B, AP-1 and NFAT. The cells were subjected to oxidative stress: an enzymatic activity (PAO) generating hydrogen peroxide, irradiation and four concentrations of hydrogen peroxide administered directly to the cells. None of these stresses affected lymphocyte viability, which remained at 95%, as determined by trypan blue exclusion. Figure 1 demonstrates the effect of oxidative stress on NF κ B DNA-binding in T lymphocytes. While unstimulated cells (Unst.) did not express NF κ B DNA binding, stimulated cells (Stim.) did express this activity, and the interaction was specific, as shown by its prevention in the presence of a specific competitor (an excess of unlabeled NF κ B DNA, Stim. + Comp.). All the types and levels of oxidative stress we employed completely abolished the induction of NF κ B DNA-binding in stimulated T lymphocytes.

As can be seen in Figs 2 and 3, protein binding activities to two other DNA sequences (AP-1 and NFAT) were only expressed in stimulated T cells and were abolished by exposing the cells to oxidative stress, similar to the results obtained with the NF κ B sequence.

Lipid peroxide determination in lymphocytes exposed to oxidative stress

Since the goal of these studies was to develop markers of oxidative stress in lymphocytes, we measured lipid peroxidation as a

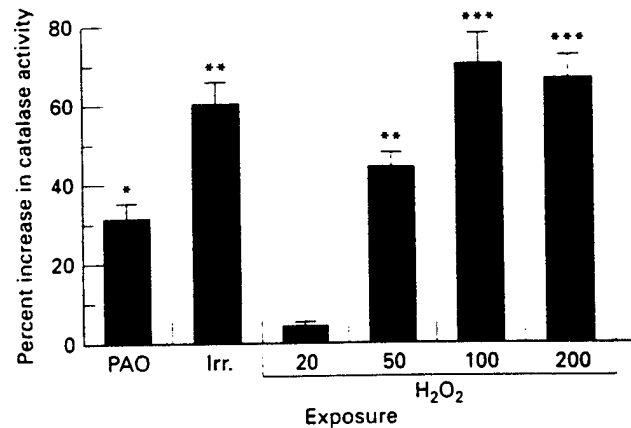


Fig. 4. Enhancement of catalase activity by oxidative stress. T cells were exposed to polyamine oxidase (PAO, for 2 days at 5×10^{-4} U/ml + spermidine at $5 \mu\text{M}$, and then washed and incubated for 2 h in fresh medium), or irradiation (Irr., for 5 min at 6 Gy and then incubated for 2 h in fresh medium), or hydrogen peroxide (H_2O_2 at the indicated concentration, μM , for 2 h and then washed and incubated for 2 h in fresh medium). Cells were lysed and cytosolic catalase activity was measured by following kinetically the decomposition of hydrogen peroxide in a spectrophotometer, and calculated per protein concentration. Catalase activity in untreated cells was 1.2 ± 0.09 ($\text{sec}^{-1} \cdot \text{mg protein}^{-1}$). Results shown are the increase in catalase activity in stressed cells over untreated cells \pm s.d. The increase in catalase activity was significant at * $P < 0.01$; ** $P < 0.005$; *** $P < 0.0005$.

biochemical parameter of exposure to oxidants. The basal level of lipid peroxides in T lymphocytes was 2.4 ± 0.7 nmol lipid hydroperoxides/mg protein. Only treatment with hydrogen peroxide at $20 \mu\text{M}$ for 2 h induced a rise in cellular peroxides to 11.9 ± 1.8 nmol lipid hydroperoxide/mg protein ($P < 0.0005$), while the other treatments (hydrogen peroxide at 50 – $200 \mu\text{M}$, polyamine oxidase and irradiation) did not induce any rise in the levels of lipid peroxides above basal levels. A possible explanation of these findings is that the direct biochemical damage was repaired within 2 h after the exposures. The lowest concentration of hydrogen peroxide may not have been sufficient to induce appropriate levels of antioxidant defences, allowing the lipid peroxidation to be detected. To investigate this possibility, levels of the major antioxidative enzyme, catalase, were measured in T lymphocytes exposed to oxidative stress.

Catalase determination in lymphocytes exposed to oxidative stress
Every oxidative stress exposure, except for hydrogen peroxide at $20 \mu\text{M}$, induced a significant rise in cellular catalase activity above the basal level (Fig. 4).

DISCUSSION

Three modes of oxidative stress—hydrogen peroxide, polyamine oxidase activity generating hydrogen peroxide gradually, and irradiation—suppressed the activation-dependent DNA-binding activities of three transcription factors, NF κ B, AP-1 and NFAT, in human peripheral blood T lymphocytes. Only exposure to hydrogen peroxide at $20 \mu\text{M}$ generated measurable lipid peroxidation products in T lymphocytes, while this was the only exposure that did not induce an increase in cellular catalase activity.

We have previously reported [8] that exposure to polyamine oxidase results in suppression of transmembrane signal transduction in human peripheral blood T lymphocytes. This leads to

suppression of the activation-dependent expression of transcription factors in the nucleus, and finally to inhibition of the transcription of the IL-2 gene. In the current study, we compared the effects of different types and levels of oxidative stress on nuclear signal transduction in exposed human lymphocytes. Three transcription factor DNA-binding activities were suppressed in T lymphocytes by every condition of oxidative stress employed. The fact that we did not detect a dose-response dependence in the suppression of transcription factor activity by hydrogen peroxide suggests that suppressing early signalling events by oxidative stress [8] results in an all-or-none effect on distal signalling steps in the nucleus.

The DNA-binding activities of NF κ B and AP-1 are induced upon exposure to oxidants [16–18]. The apparent contradiction with our results may be resolved by recognizing that in our system (but not in the other studies mentioned) cells were incubated for 2 h in fresh medium after the exposures, followed by stimulation for 6 h, and only then were DNA-binding activities determined. Therefore, we are assessing the effects of oxidative stress on T cell mitogenic activation rather than the direct effect on transcription factor activities. We have previously found that oxidative stress suppresses early signal transduction steps, protein tyrosine phosphorylation and calcium mobilization [8]. Therefore, the eventual suppression of transcription factor activities may actually reflect early effects of oxidative stress on lymphocyte transmembrane signal transduction. In this context, the decline in IL-2 production by human T lymphocytes from aged persons in response to *in vitro* stimulation is associated with impaired activation of AP-1 and NFAT [19]. In view of the oxygen radical-related theory of ageing, this is potentially an example of T cell suppression at the signal transduction level by oxidative stress *in vivo*.

We found that radiation suppressed the expression of DNA-binding activities in activated T lymphocytes. Radiation of human lymphocytes *in vitro* was previously found to suppress constitutive surface marker expression [20] and enhance micronuclei occurrence following stimulation [21].

Since NF κ B and AP-1 were recently found to be the most important IL-2 cis-regulatory elements in normal T cells [22], and we have shown that the acute and chronic modes of oxidative stress used in this study suppress IL-2 production by human lymphocytes [7,9], our results strongly suggest that suppression of transcription factor function caused by inducing oxidative stress in human lymphocytes contributes to down-regulation of IL-2 production and cellular activation. IL-2 is a major growth factor regulating T lymphocyte proliferation [23]. Therefore, suppression of nuclear signalling events that control IL-2 expression reflects not only exposure but also the detrimental effect of exposure to oxidative stress inducers.

Direct measurements of lipid hydroperoxides did not detect increased levels following oxidative stress, except for exposure of T lymphocytes to the lowest concentration of hydrogen peroxide. The same hydrogen peroxide concentration (20 μ M) was the only one from among the oxidative stress exposures employed in the current study that did not induce a rise in catalase activity. Therefore, we suggest that when the oxidative stress induces an anti-oxidative response, i.e. catalase activity, lipid peroxidation is not detectable. PAO did not induce lipid peroxidation, although the enzymatic activity generates only 5 μ M H₂O₂, because the intensity of the cellular stress is determined not only by the concentration of the oxidant but also by the duration of the exposure (in the case of PAO, 2 days). Accordingly, PAO exposure induced an increase in cellular catalase activity.

On the other hand, all three modes of oxidative stress resulted in suppression of cellular function that was clearly evident even 8–26 h after the exposures, as judged by transcription factor activities. Our results suggest that a functional parameter (nuclear signal transduction) is much more sensitive than a structural parameter (lipid peroxidation) as a marker of oxidative damage to human blood lymphocytes.

These results suggest that transcription factor functions can potentially be used as markers of blood lymphocyte exposure to oxidants (including hydrogen peroxide and hydroxyl radicals) which generate either acute or chronic stresses. Our studies were conducted with human PBL, which are readily available, and should therefore be amenable to development into population-based markers of environmental exposure to oxidants. *Ex vivo* peripheral blood cells can also be used for follow up of exposed individuals.

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Ozone-induced IL-8 expression and transcription factor binding in respiratory epithelial cells

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Jaspers, I., E. Flescher, and L. C. Chen. Ozone-induced IL-8 expression and transcription factor binding in respiratory epithelial cells. *Am. J. Physiol.* 272 (*Lung Cell. Mol. Physiol.* 16): L504–L511, 1997.—Ozone, one of the most reactive oxidant gases to which humans are routinely exposed, induces inflammation in the lower airways. The airway epithelium is one of the first targets that inhaled ozone will encounter, but its role in airway inflammation is not well understood. Expression of inducible genes involved in the inflammatory response, such as interleukin (IL)-8, is controlled by transcription factors. Expression of the IL-8 gene is regulated by the transcription factors nuclear factor (NF)- κ B, NF-IL-6, and possibly activator protein-1 (AP-1). Type II-like epithelial cells (A549) were grown on a collagen-coated membrane and exposed in vitro to 0.1 ppm ozone or air. Exposure to ozone induced DNA-binding activity of NF- κ B, NF-IL-6, and AP-1. IL-8 mRNA and IL-8 protein levels were also increased after ozone exposure. These results link ozone-induced DNA-binding activity of transcription factors and the production of IL-8 by epithelial cells thus demonstrating a potential cellular cascade resulting in the recruitment of inflammatory cells into the airway lumen.

nuclear factor- κ B; nuclear factor-interleukin-6; activator protein-1; A549 cells

OZONE IS a common urban air pollutant to which humans are routinely exposed. The National Ambient Air Quality Standard for ozone, 0.12 ppm for a daily 1-h average, is exceeded in more than 60 cities of the United States (38). Laboratory animal and human clinical studies have demonstrated that ozone causes reversible decrements in pulmonary function, increased permeability of the epithelium, influx of inflammatory cells, impaired pulmonary defense capacity, and tissue damage (21). The morphological and biochemical changes measured after ozone exposure result from the direct effects of the interaction of ozone (or its derived products) with cells and the inflammatory response against that initial damage (38).

An important cellular mechanism by which the lung reacts to inhaled noxious gases or particles is the recruitment of inflammatory cells, especially neutrophils, from the vasculature into the airway lumen. These neutrophils release soluble factors, including proteolytic enzymes and reactive oxygen intermediates (ROI), which can initiate changes in lung function and morphology (17). Increased numbers of infiltrated neutrophils in the bronchoalveolar lavage fluid (BALF) of humans exposed to ambient concentrations of ozone (0.1 ppm for 6.6 h) were observed 18 h postexposure (12), whereas markers of airway inflammation in the BALF of exercising individuals exposed to 0.4 ppm for 2 h could be observed as early as 3 h postexposure (30).

It is not yet known which cell type initiates the lower airway inflammatory response after ozone exposure. The pulmonary epithelium, comprised of several different cell types, is one of the first targets that inhaled ozone will encounter and could therefore be a plausible initial mediator of pulmonary inflammation. Specifically, epithelial cells in the proximal alveolar region are susceptible to damage by inhaled ozone (6). Alveolar type II cells play an important role in defense mechanisms of the respiratory tract by acting as a physical barrier to inhaled agents and as the main source of pulmonary surfactant (35). More recently, the airway epithelium has been implicated as an "effector" tissue responding to exogenous stimuli by releasing a variety of cytokines (1), such as interleukins (IL)-1, -3, -6, and -8, granulocyte macrophage colony-stimulating factor, granulocyte colony-stimulating factor (G-CSF), and tumor necrosis factor- α (TNF- α ; see Ref. 11). IL-8, a potent neutrophil chemotactic factor (5), is a potential mediator of pulmonary inflammation.

DNA binding of transcription factors is a necessary step in the expression of inducible genes involved in inflammatory and immune responses. Oxidative stress appears to be an important regulator of IL-8 gene expression (10), which has been shown to be under the synergistic control of nuclear factor (NF)- κ B and NF-IL6 sites in its promoter region (18). An NF-IL-6-like sequence was identified between nucleotides -94 and -81, whereas NF- κ B binds to a region between nucleotides -80 and -71 (18). Activation of NF- κ B and NF-IL-6 DNA-binding activity is thought to occur through the induction of signaling cascades involving ROI and protein kinases (28, 36). An activator protein-1 (AP-1) sequence was also identified, but its activity may be dispensable (18). DNA-binding activity of AP-1 is regulated by a redox mechanism involving a conserved cysteine residue in the DNA-binding domain and/or phosphorylation of the AP-1 subunits Fos and Jun (9). The role of ozone in the activation of protein kinases and the induction of DNA-binding activity of NF- κ B, NF-IL-6, and AP-1 is currently not known.

In this study, we investigated whether in vitro exposure of A549 cells, a human alveolar type II-like cell line, to a low level of ozone induces the DNA-binding activity of the transcription factors NF- κ B, NF-IL-6, and AP-1 and whether this is associated with ozone-induced activation of IL-8 gene transcription and translation. Furthermore, we examined whether these cells display polarity in their release of IL-8. Our results demonstrated that in vitro exposure of A549 cells to 0.1 ppm ozone induced the activity of the transcription factors NF- κ B, NF-IL-6, and AP-1 and increased the expression of IL-8 mRNA and production of IL-8. The

release of IL-8 was initially and predominantly toward the apical side, supporting the proposed mechanism of neutrophil movement along a chemotactic gradient (5) toward the airspace lumen.

MATERIALS AND METHODS

Cell lines and cell culture. A human pulmonary type II epithelial-like cell line (A549 from American Type Cell Culture, Rockville, MD), derived from a patient with alveolar cell carcinoma of the lung, was used in this study. These cells retain features of type II alveolar epithelial cells, including cytoplasmic multilamellar inclusion bodies, but cannot be definitively characterized as of type II origin or function (19). A549 cells were cultured in F-12K media plus 10% fetal bovine serum (FBS; GIBCO-BRL, Gaithersburg, MD) plus 1% penicillin and streptomycin (GIBCO-BRL).

In vitro exposure. A549 cells were grown on Vitrogen-coated (Collagen, Palo Alto, CA) Costar clear transwells (25 mm diameter, 0.4 μ m pore size; Costar, Cambridge, MA) until a confluent monolayer was established ($\sim 2 \times 10^6$ cells/well). To avoid interference of serum components with possible ROI-mediated ozone effects, cells were cultured in phenol red-free F-12 nutrient mixture (Ham's; GIBCO-BRL) without FBS 1 h before and throughout the exposure. Just before exposure, the apical media was aspirated while 2 ml media remained in the basolateral compartments to supply cells with nutrients. The cell monolayers were kept at 37°C and exposed to preheated/prehumidified 0.1 ppm ozone, balanced with 5% CO₂, or exposed to incubator air (which hereafter will be called air-exposed cells) for 2.5 or 5 h. This ozone concentration and exposure duration are frequently achieved in the ambient air of southern California (21). Ozone was produced by passing 0.5% O₂ (in argon carrier) through an ozonizer (Sander), and the exposure concentration was monitored continuously using an Ozone Analyzer (model 8810; Monitor Labs, San Diego, CA). Unexposed incubator control cells remained covered by F-12 media on the apical side throughout the exposure period. To evaluate whether the effects seen after ozone exposure were unique to ozone or were caused by a nonspecific state of oxidative stress, cells received a bolus addition of 0.1, 0.5, and 1 mM H₂O₂, which remained in the apical compartment for 5 h.

Immediately after exposure, cells were removed from the membranes using 0.1% trypsin-EDTA (GIBCO-BRL), and viability was assessed using the trypan blue exclusion method.

Electrophoretic mobility shift assay. Nuclear factors for the mobility shift assay were prepared from A549 cells immediately after exposure as described by Flescher et al. (14). Briefly, cells were washed with phosphate-buffered saline. Extraction buffer made of 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 10 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), and seven protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 μ g/ml aprotinin, 5 μ g/ml antipain, 100 μ M benzamidine, 5 μ g/ml leupeptin, 5 μ g/ml pepstatin, and 5 μ g/ml soybean trypsin-chymotrypsin inhibitor, pH 7.9) was added to the monolayers, and the cells were removed by manual scraping with a rubber policeman and transferred to a microcentrifuge tube. The cells were allowed to swell on ice for 15 min, and Nonidet P-40 at 0.625% was added. The tube was vortexed and centrifuged for 30 s in a microcentrifuge. The nuclear pellet was resuspended in buffer containing 20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and the seven previously listed protease inhibitors. The tube was vigorously rocked on ice for 15 min on an orbital

shaker platform, and the nuclear extract was centrifuged for 5 min in a microcentrifuge. The supernatant contained the nuclear extracts. Protein concentrations of the nuclear extracts were determined using the Bradford method (Bio-Rad protein assay kit). Oligonucleotides containing the NF- κ B and AP-1 sequences (Promega, Madison, WI) were labeled using [γ -³²P]ATP and T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The oligonucleotide containing the NF-IL-6 consensus sequence (5'-TTCAACCTGTTTCGCAGT-TTCTCGAGGAATCA-3'; see Ref. 8) was labeled using [α -³²P]CTP and Klenow enzyme (Boehringer Mannheim, Indianapolis, IN). Four micrograms of nuclear extract, 20,000 counts/min labeled probe, and 2 μ g poly dI/dC were mixed in a total volume of 20 μ l. The mixture was incubated for 60 min on ice, loaded on a 4% polyacrylamide gel, and subjected to electrophoresis at 20 mA for 1.5–2 h. The gel was dried and autoradiographed on Kodak XAR-5 film at -70°C for 1–3 days.

RNA analysis. RNA was extracted at 0, 4, and 16 h postexposure using RNazol (Biotecx, Houston, TX). The amount of RNA present was determined by spectrophotometric absorbance at 260 nm. RNA (15 μ g) was loaded onto a 1.5% agarose gel and subjected to electrophoresis at 15–20 volts overnight. The electrophoresed RNA was transferred onto membrane filters (Nytran; Schleicher & Schuell, Keene, NH) and fixed by ultraviolet cross-linking. Membrane-bound RNA was prehybridized and then hybridized to [³²P]cDNA probe (IL-8 cDNA; R & D Systems, Minneapolis, MN) at 52°C in 1 \times sodium chloride-sodium phosphate-EDTA (SSPE), 2 \times Denhardt's, 0.1% sodium dodecyl sulfate (SDS), and 0.1 mg/ml denatured tRNA and salmon sperm DNA. As control, 28S cDNA (Clontech Laboratories, Palo Alto, CA) was radiolabeled and hybridized as described above. After hybridization, the filters were washed at a final stringency of 5 \times SSPE-0.1% SDS at 52°C and exposed to Kodak X-OMAT S film at -70°C for 1–3 days. Autoradiogram signal strengths of hybridized mRNA were quantified using a laser densitometer (BioImage; Millipore, Ann Arbor, MI). All IL-8 mRNA levels were normalized to 28S expression and expressed as degree of induction over control mRNA isolated from incubator control cells at time 0.

Measurement of IL-8 levels. Immediately after the exposure period, all media were aspirated, and 1 ml fresh phenol red-free F-12 nutrient mixture was added to the apical and basolateral sides. Cells were cultured for an additional 4, 16, and 24 h, and at each time point the conditioned culture media was collected for measurement of IL-8 levels. Release of IL-8 by epithelial cells into the apical and basolateral compartments was assayed with a human IL-8 enzyme-linked immunosorbent assay kit (R & D Systems), according to the instructions given by the manufacturer. Briefly, the IL-8 present in the samples reacts with an immobilized murine monoclonal antibody against IL-8. An enzyme-linked polyclonal antibody specific for IL-8 reacts with the IL-8 bound to the monoclonal antibody. Addition of the enzyme substrate develops color in proportion to the bound IL-8. Any unbound proteins are washed away with a buffered surfactant solution. To assess total IL-8 released by A549 cells, the amounts of IL-8 secreted into the apical and basolateral compartments were combined and expressed as picograms IL-8 per well.

Statistical analysis. All data were analyzed relative to control. This was done using a two-way analysis of variance to test for exposure and postexposure time effects. The Fisher's post hoc test was used to determine if the exposed groups were different from one another. Three Transwells per exposure group were used in this study.

RESULTS

DNA-binding activity of NF- κ B, NF-IL-6, and AP-1.

Figure 1 shows that neither exposure to air or 0.1 ppm ozone nor treatment with 1 mM H_2O_2 for 5 h caused a significant decrease in viability of A549 cells compared with unexposed control cells. The effects of ozone exposure on the DNA-binding activity of NF- κ B, NF-IL-6, and AP-1 in A549 cells are shown in Fig. 2. Analysis of nuclear factors isolated from A549 cells exposed to 0.1 ppm ozone for 5 h using the mobility shift assay showed the induction of the DNA-binding activity of all three transcription factors. Exposure to air had no effect on the activity of NF- κ B and NF-IL-6 but slightly induced the DNA-binding activity of AP-1. Nuclear factors isolated from unexposed control cells also showed a weak band of DNA-binding activity of AP-1 (data not shown), similar to the activity of nuclear extracts from air-exposed cells. These results suggest that AP-1 may be constitutively present in A549 cells. In addition, treatment with H_2O_2 induced the DNA-binding activity of NF- κ B (Fig. 2A, lane 2) but did not induce NF-IL-6 DNA binding (Fig. 2B, lane 2). Specificity of the transcription factor DNA-binding activity was examined by adding an excess of the specific unlabeled oligonucleotide to the reaction mixture. The addition of 50 \times nonradiolabeled oligonucleotide successfully competed for the DNA binding of the respective transcription factor.

Induction of IL-8 transcription after ozone exposure.

The induction of the DNA-binding activity of transcription factors that control IL-8 gene expression (22) suggested that ozone exposure may affect IL-8 mRNA levels in A549 cells. To examine the effect of ozone and air exposure on IL-8 mRNA levels, RNA was isolated at

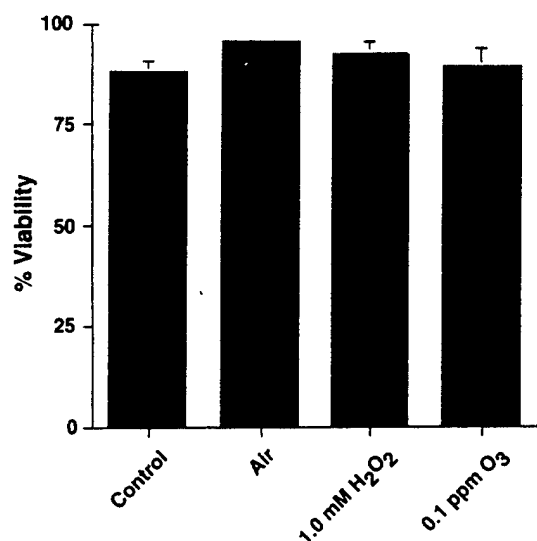


Fig. 1. Effects of air and ozone exposure or treatment with 1 mM H_2O_2 on cell viability. Exposure to ozone or air or treatment with H_2O_2 did not decrease cell viability compared with control cells. After exposure to 0.1 ppm ozone or air or treatment with 1 mM H_2O_2 for 5 h, cell viability was assessed by trypan blue exclusion. Values shown represent means \pm SE of 3 separate experiments. Error bar for air-exposed cells is too small to be shown.

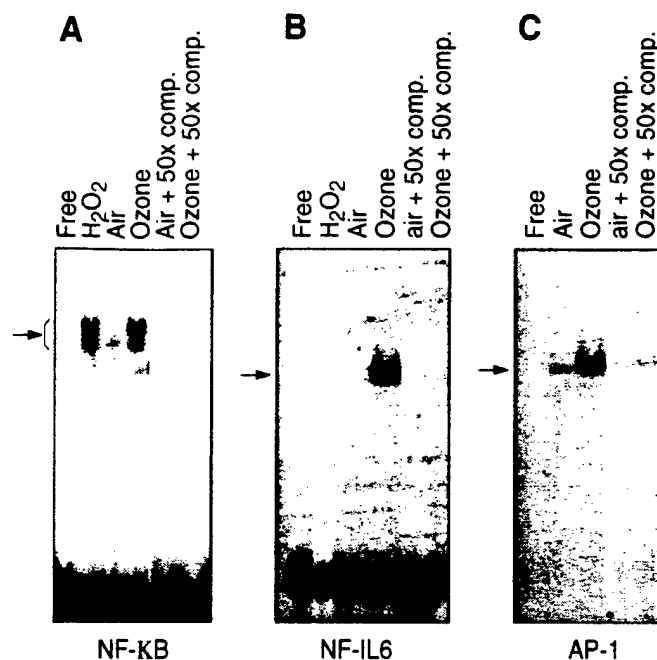


Fig. 2. Exposure to ozone induces nuclear factor (NF)- κ B, NF-IL-6, and activator protein-1 (AP-1) DNA-binding activity. A549 cells were exposed to air or 0.1 ppm ozone or treated with 1 mM H_2O_2 for 5 h, nuclear extracts were prepared, and 4 μ g protein were incubated with 32 P-labeled NF- κ B (A), NF-IL-6 (B), and AP-1 (C) sequences and then electrophoresed. Lanes were free (DNA without nuclear extract) or loaded with air (nuclear extract from air-exposed cells), ozone (nuclear extracts from ozone-exposed cells), air + 50 \times comp (same as air plus 50 \times excess of unlabeled probe), or ozone + 50 \times comp (same as ozone plus 50 \times excess unlabeled probe). In A and B, lane 2 is H_2O_2 (nuclear extracts isolated from H_2O_2 -treated cells). Gels were dried and autoradiographed. Arrows mark specific DNA-protein complexes.

0, 4, and 16 h postexposure. As illustrated in Fig. 3, exposure to 0.1 ppm ozone for 5 h induced a significant increase in IL-8 mRNA levels within 4 h after exposure compared with air-exposed cells. At 16 h postexposure, the mRNA levels in the ozone-exposed cells decreased. Exposure to air also elevated expression of the IL-8 gene in A549 cells compared with unexposed control cells but was significantly lower than in ozone-exposed cells.

Comparison of ozone- and H_2O_2 -induced expression of IL-8. To investigate whether the changes seen after ozone exposure are caused by general oxidative stress or some other more ozone-specific phenomena, we treated cells with various concentrations of H_2O_2 for 5 h. The autoradiograph in Fig. 4A compares IL-8 mRNA isolated from H_2O_2 -treated and ozone-exposed A549 cells 4 h postexposure. Treatment with various concentrations of H_2O_2 did not increase IL-8 mRNA levels, in contrast to what is seen after exposure to ozone. Similarly, Fig. 4B shows that IL-8 production 4 h after exposure was significantly higher in ozone-exposed cells compared with unexposed control cells, air-exposed cells, or H_2O_2 -treated cells. Neither air exposure nor treatment with various concentrations of H_2O_2 significantly increased IL-8 production compared with

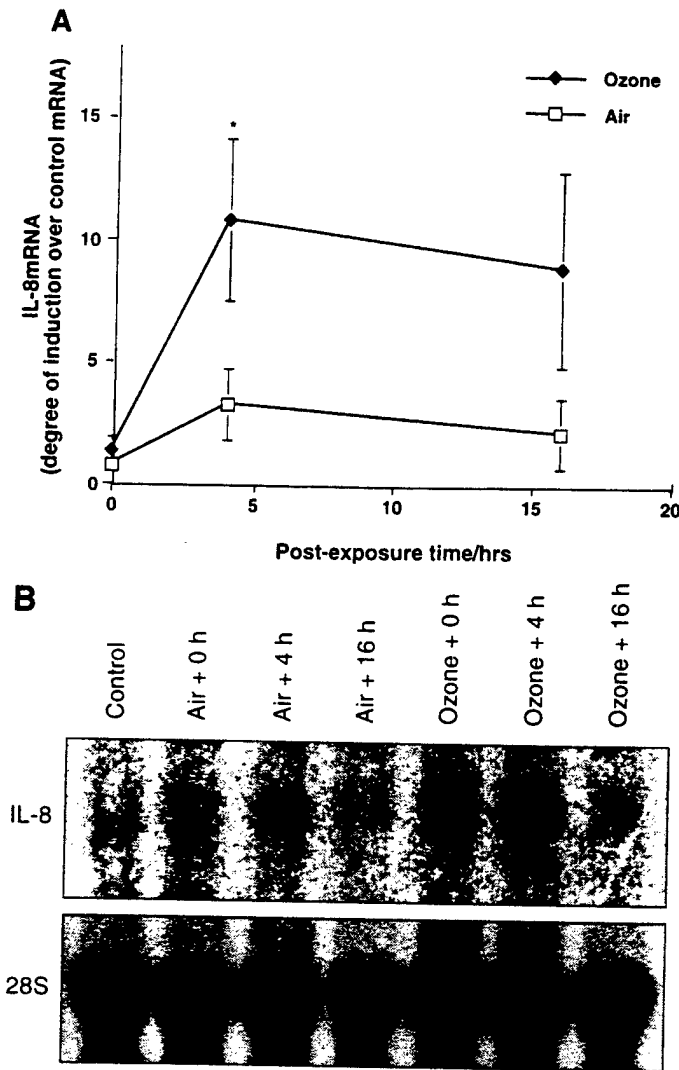


Fig. 3. Exposure to ozone increases interleukin (IL)-8 mRNA levels. RNA was isolated from A549 cells at 0, 4, and 16 h after exposure to 0.1 ppm ozone or air for 5 h. A: IL-8 mRNA levels were normalized to 28S rRNA and expressed as percent of control cells. Values shown represent means \pm SE of 3 individual experiments. *Significantly different from air-exposed cells; $P < 0.05$. B: representative autoradiograph showing hybridization to IL-8 (top) and 28S (bottom) cDNA. Northern blot analysis shows that exposure to ozone induces expression of IL-8 gene within 4 h after exposure.

unexposed control cells. This suggests that exposure to air or treatment with H_2O_2 had no significant effect on the regulation of IL-8 synthesis beyond the documented spontaneous production of IL-8 by A549 cells (3), as detected by us in control cells.

Effects of exposure duration on IL-8 expression. To examine whether the effect of ozone on the expression of IL-8 was dose dependent, we analyzed IL-8 mRNA and protein levels after 2.5- and 5-h exposures to 0.1 ppm ozone. Figure 5, A and B, compares expression of IL-8 after 2.5- and 5-h exposures. Figure 5 shows that exposure of A549 cells to 0.1 ppm ozone for 5 h induced a significantly higher expression of IL-8 than exposure for 2.5 h. At both time points, IL-8 production was

significantly higher in ozone-exposed cells than in air-exposed cells.

Comparison of IL-8 levels in the apical and basolateral compartments. Because airway epithelial cells in vivo are thought to possess polarity in their morphology and release of mediators, we examined next whether the ozone-induced accumulation of IL-8 was different in the apical versus basolateral compartments. Figure 6

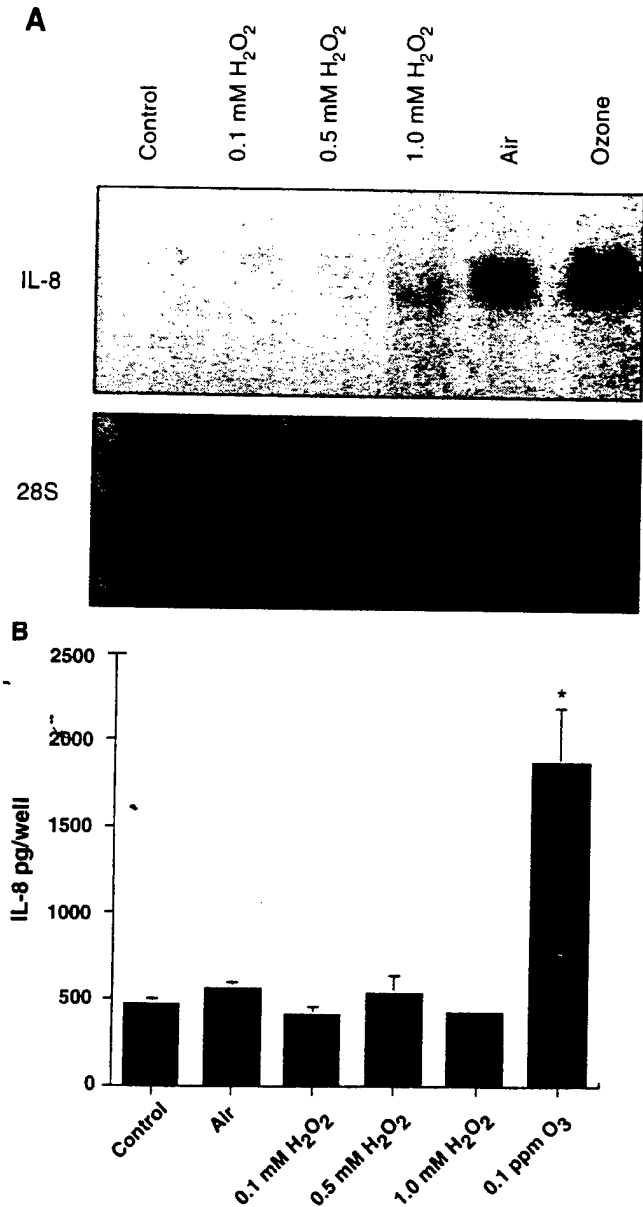


Fig. 4. Comparison of ozone- and H_2O_2 -induced effects on IL-8 expression. A: representative autoradiograph showing hybridization of RNA isolated from A549 cells 4 h postexposure or posttreatment to IL-8 (top) and 28S (bottom) cDNA. Northern blot analysis shows that 5 h of exposure to ozone induces greater expression of IL-8 gene than either exposure to air or treatment with 0.1, 0.5, or 1.0 mM H_2O_2 for 5 h. B: analysis of IL-8 content in conditioned media 4 h after exposure shows that ozone induces significantly higher release of IL-8 than exposure to air or treatment with H_2O_2 . *Significantly different from control, air, or treatment with 0.1, 0.5, or 1.0 mM H_2O_2 ; $P < 0.05$. Values are means \pm SE.

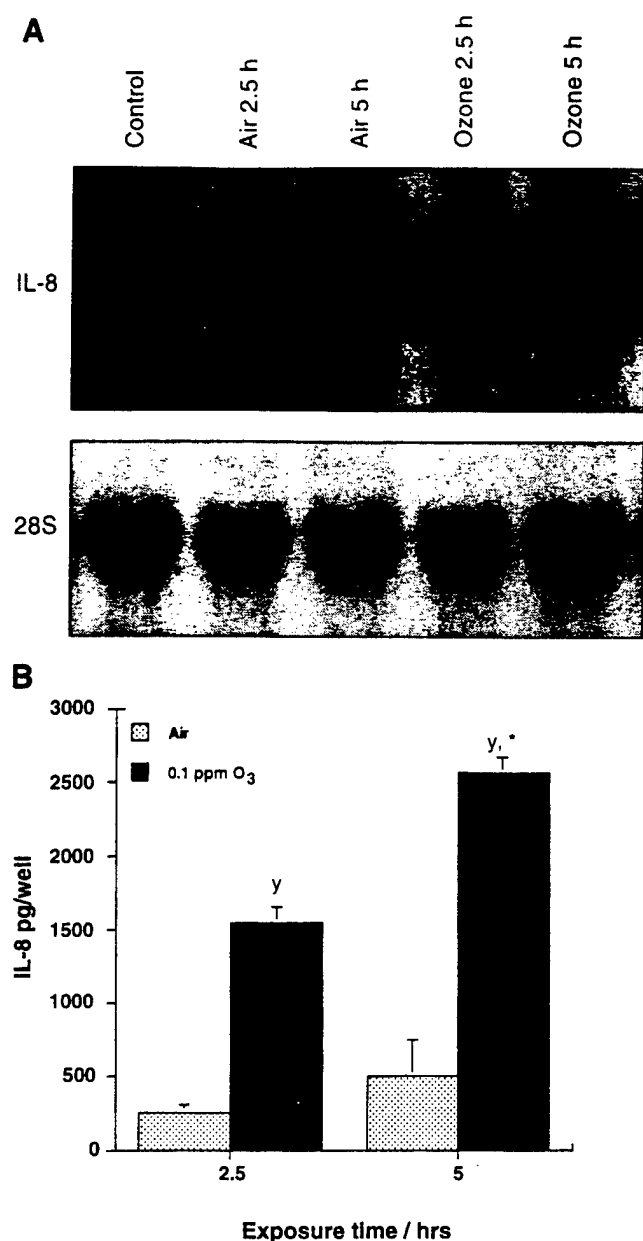


Fig. 5. Effects of exposure duration on IL-8 expression. A: representative autoradiograph showing hybridization of RNA isolated from A549 cells 4 h postexposure to IL-8 (top) and 28S (bottom) cDNA. Northern blot analysis shows that 5 h of exposure to ozone induces greater expression of IL-8 gene than exposure for 2.5 h. B: analysis of IL-8 content in conditioned media 4 h after exposure shows that exposure to 0.1 ppm ozone for 5 h induces significantly higher release of IL-8 than exposure for 2.5 h and that ozone induces greater expression of IL-8 than air exposure independent of exposure duration. ^ySignificantly different from air-exposed cells; ^{*}significantly different from 2.5-h exposure; $P < 0.05$. Values are means + SE.

shows that a significant rise in IL-8 in the apical compartments could be observed as early as 4 h after exposure, whereas this did not occur until 16 h postexposure in the basolateral compartments. At 24 h postexposure, IL-8 content in both apical and basolateral compartments decreased in ozone-exposed cells. The greatest difference between apical and basolateral IL-8 content was seen at 4 h postexposure. This difference

was still significant at 16 h postexposure. Air exposure did not significantly increase IL-8 release into the apical or basolateral compartments compared with unexposed control cells.

DISCUSSION

The cellular mechanisms that induce cytokine production in lung cells after pollutant exposures are still unclear. Previous studies have suggested that IL-8 synthesis, which is under the synergistic control of the transcription factors NF- κ B and NF-IL-6, and possibly AP-1 (18), is initiated by oxidative stress (10). We therefore investigated whether exposure of respiratory epithelial cells to the oxidant pollutant ozone induces changes in the DNA-binding activities of NF- κ B, NF-IL-6, and AP-1 and whether these changes are reflected in increased IL-8 mRNA and IL-8 protein levels. Our results demonstrated that in vitro exposure of A549 cells to an environmentally relevant concentration of ozone (21) induced the DNA-binding activity of transcription factors responsible for the control of IL-8 gene expression. The activation of NF- κ B and NF-IL-6,

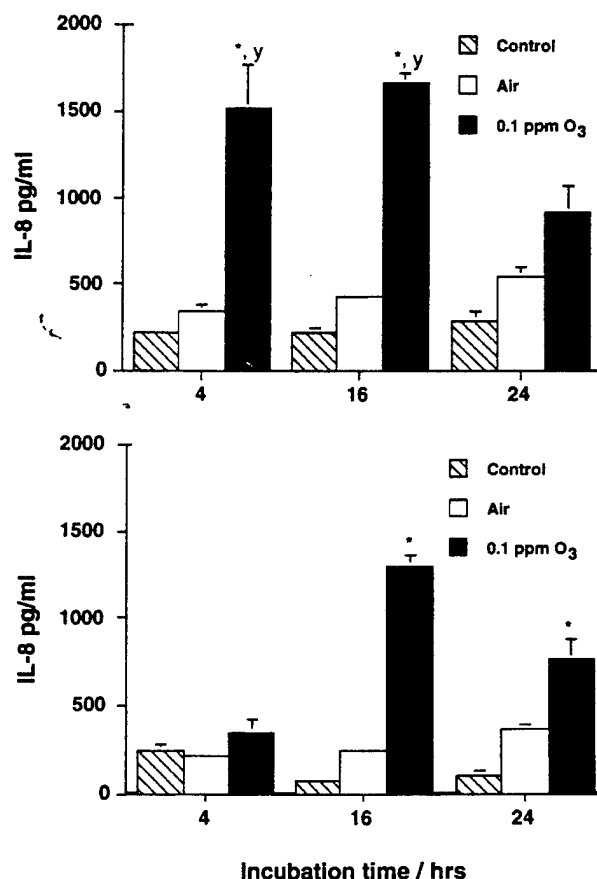


Fig. 6. Comparison of IL-8 levels in apical (top) and basolateral (bottom) media. Conditioned media was collected at 4, 16, and 24 h after exposure to air or 0.1 ppm ozone for 5 h. Enzyme-linked immunosorbent assay of IL-8 production in apical and basolateral media shows that release of IL-8 is initially only toward the apical side. ^{*}Significantly different from air-exposed cells; ^ysignificantly different from basolateral media; $P < 0.05$. Values shown represent means + SE of 3 individual experiments. Some error bars of IL-8 levels in air-exposed cells are too small to be shown.

which are essential in the expression of other inflammatory cytokines, such as IL-6, G-CSF, and TNF- α (2, 24), could comprise a proximal step in the ozone-induced inflammatory response at a concentration that is not overtly cytotoxic.

Other studies investigating pollutant-induced activities of transcription factors and expression of inflammatory cytokines concur with our findings regarding the ozone-induced activation of the transcription factors NF- κ B and NF-IL-6 and the production of IL-8 by human pulmonary epithelial cells. For example, the DNA-binding activities of NF- κ B and NF-IL-6 as well as the production of IL-8 were induced in asbestos-exposed pulmonary epithelial cells, and the response was thought to be mediated through asbestos-induced oxidative stress (31). In another study, the activation of NF- κ B in lung tissue and the expression of the chemokine cytokine-induced neutrophil chemoattractant were observed in rats exposed to a high level of ozone (16). It is possible that the induction of DNA-binding activity of transcription factors, specifically NF- κ B and NF-IL-6, may result in the generation of other inflammatory cytokines, such as G-CSF, TNF- α , and IL-6, by pulmonary cells. Therefore, activities of these transcription factors may constitute a common and early step in pollutant-induced inflammatory processes.

In our study, we observed dose-dependent increases in the transcription of the IL-8 gene and production of IL-8 in ozone-exposed respiratory epithelial cells. Although these changes correlated with increased transcription factor activities, further experiments, such as using the recently developed inhibitor for nuclear translocation of NF- κ B (20) or NF-IL-6 knockout mice (33), are necessary to establish the dependency of ozone-induced IL-8 release and transcription factor binding. Nevertheless, the rise in NF- κ B and NF-IL-6 DNA-binding activities and levels of IL-8 mRNA after the same exposure conditions, as well as the dependency of IL-8 transcription on the activities of NF- κ B and NF-

IL-6 shown by others (18, 22), suggest such a relationship.

Ozone is thought to exert its toxic effects through the formation of ROI at or near the epithelial membrane (26). It is likely that pulmonary cells respond specifically to the membrane-associated ROI production rather than a general state of oxidative stress, such as that produced by H₂O₂. Indeed, as shown in our study, while inducing the DNA-binding activity of NF- κ B, similar to that shown by others (29), apical stimulation of A549 cells with 1 mM H₂O₂ failed to induce the DNA-binding activity of NF-IL-6, a transcription factor essential for the expression of IL-8 (18, 22). Furthermore, IL-8 mRNA and IL-8 protein levels were significantly lower in H₂O₂-treated cells than in ozone-exposed cells. These results indicate that the ozone-induced signaling cascade is initiated, at least partially, by an ozone-specific mechanism rather than by a general state of oxidative stress. Evidence for the existence of an ozone-specific mechanism is supported by recent findings that lipid ozonization products (LOP), formed through the reaction of ozone with membrane lipids, may be responsible for the effects seen after ozone exposure (27).

The airway epithelium is known to possess polarity in its morphology. Primary human tracheal epithelial cells grown on membranes with a gas-liquid interface retain that polarity in vitro, displaying ciliogenesis and mucus production toward the apical side (15). Interestingly, the results of our study indicate that A549 cells retained polarity in their release of IL-8 after exposure to ozone. Comparison of the apical and basolateral conditioned media of ozone-exposed A549 cells showed that the IL-8 release was mainly toward the apical side, hence creating a concentration gradient. Our results are in contrast to a study published by Devlin et al. (13), which showed polarity in the release of IL-6, but not IL-8, by a human bronchial epithelial cell line exposed to ozone. The discrepancy in the results could stem from differences in the cell line and exposure duration.

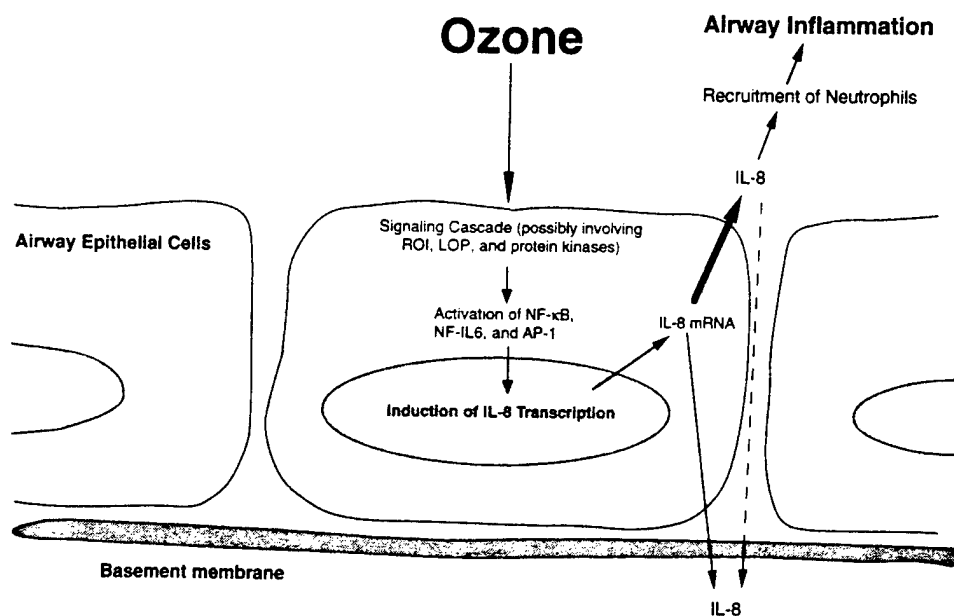


Fig. 7. Model of proposed biochemical events induced in A549 cells upon exposure to ozone, leading to airway inflammation. ROI, reactive oxygen intermediates; LOP, lipid ozonization products.

The cell line used in our study has type II-like cell characteristics, whereas an SV40-transformed bronchial epithelial cell line was used by Devlin et al. (13).

Although in vitro exposures to ozone in a cell culture system do not directly mimic in vivo exposure conditions, it is a useful model to study intracellular mechanisms of pollutant-induced injury in a homogenous cell population (3, 7, 23). Culturing respiratory epithelial cells on collagen-coated membranes, with nutrients supplied from the basolateral side, facilitates the direct exposure of these cells to gaseous pollutants on the apical side, without the influence of other cellular and biochemical reactions. Although these reactions are important parameters in the initiation of lung injury, they also make the investigation of the exact role that respiratory epithelial cells play in pollutant-induced inflammatory responses very difficult. As in other in vitro studies, one has to be careful in extrapolating the results obtained in this study to the in vivo situation, especially since the cell line used throughout this study cannot be definitively characterized as of alveolar type II cell origin or function.

In conclusion, our study showed that exposure to ozone induced the DNA-binding activities of NF- κ B, NF-IL-6, and AP-1, as well as the expression of IL-8, in a dose-dependent manner and that this response was specific to ozone and could not be induced by treatment with H₂O₂. Our results suggest that interaction of ozone with epithelial plasma membranes induced an intracellular signaling cascade, resulting in the generation of an IL-8 concentration gradient. The proposed sequence of cellular events, as illustrated in Fig. 7, is as follows. Formation of LOP or ozone-derived ROI could activate protein kinases, such as protein kinase C and tyrosine kinases (4, 27, 28, 32, 36). Upon phosphorylation, NF- κ B, NF-IL-6, and AP-1 would be able to translocate into the nucleus (9, 25, 34, 37), bind to the DNA sequence in the IL-8 promoter region, and facilitate transcription. The ozone-induced IL-8 release would be initially predominantly toward the apical side, thus creating a chemotactic gradient along which neutrophils could migrate into the airway lumen. IL-8 detected in the basolateral side may be derived from site-directed secretion or diffusion of apically released IL-8. The absence of obvious cell injury indicates that ozone could physiologically stimulate cytokine production in respiratory epithelial cells. The expression of IL-8 shown in this study lies within the time frame of neutrophil infiltration observed in humans (17). Therefore, our findings may help explain the influx of inflammatory cells into the lung lumen of humans exposed to ambient levels of ozone (12).

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Induction of Interleukin-8 By Ozone Is Mediated By Tyrosine Kinase and Protein Kinase A, But Not By Protein Kinase C

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Ozone is one of the most common air pollutants humans routinely inhale. We have previously shown that *in vitro* ozone exposure induces the DNA-binding activities of NF- κ B and NF-IL6 as well as the expression of interleukin 8 in respiratory epithelial cells. In this study, we investigated intracellular signaling steps mediating ozone-induced inflammatory mediator release. A549 cells, a type II like alveolar epithelial cell line, were exposed *in vitro* to air or 0.1 ppm of ozone in the presence of several kinase inhibitors. Exposure to ozone increased interleukin 8 expression and transcription factor activities in a protein tyrosine kinase (PTK)-dependent and protein kinase A (PKA)-dependent, yet protein kinase C (PKC)-independent manner. Furthermore, ozone-induced PTK and PKA activities but failed to induce PKC activity. In addition, our results suggest that ozone-induced PTK and PKA activities were reactive oxygen intermediate dependent and occurred in parallel, because specific inhibitors for PTK and PKA failed to block the other kinase's activity. These results indicate that PTK and PKA activities are early events in the signal transduction cascade mediating the ozone-induced activation of NF- κ B and NF-IL6 as well as the release of interleukin 8. *J. Cell. Physiol.* 177:313-323, 1998. © 1998 Wiley-Liss, Inc.

Ozone, a highly reactive oxidant gas, is one of the most ubiquitous pollutants in urban air. It has been estimated that in the United States each year over 66 million people are exposed to ozone levels that exceed the National Ambient Air Quality Standard of 0.12 ppm, as a 1-h average (Seltzer et al., 1986). Because of its oxidative reaction potential, inhaled ozone reacts with many constituents of the lung lining fluid layer and/or components of cellular membranes such as polyunsaturated fatty acids (Pryor and Church, 1991) to form reactive oxygen intermediates (ROIs) or lipid ozonization products (LOPs), which may be responsible for the inflammatory response seen after ozone exposure (Pryor et al., 1995). However, the exact mechanisms by which ozone induces its toxic effects are not yet known.

A hallmark of ozone toxicity is infiltration of neutrophils into the airway lumen (Seltzer et al., 1986; Devlin et al., 1991). The recruitment of inflammatory cells to the site of injury is a multistep process. The expression of chemoattractants such as interleukin 8 (IL-8) has been recognized to be important in the movement of neutrophils across the epithelium into the airway lumen (Smart and Casale, 1993). Studies conducted by us and others have established the capacity of respiratory epithelial cells to release IL-8 in response to inflammatory stimulants (Devlin et al., 1994; Bittleman and Casale, 1995; Jaspers et al., 1997), including ozone. Other stimulants such as cytokines, phorbol esters, osmotic pressure, retinoic acid, and ROIs (Baggiolino and

Clark-Lewis, 1992; Zhang et al., 1992; Chabot-Fletcher et al., 1994; Shapiro and Dinarello, 1995) are also known to stimulate the production of IL-8 by respiratory epithelial and other cells. It appears that, depending on stimulant and cell type, there are multiple signaling cascades leading to IL-8 synthesis. For example, IL-8 production is protein kinase C (PKC) dependent after stimulation of keratinocytes or fibroblasts with phorbol esters (Chabot-Fletcher et al., 1994; Zhang et al., 1992), but PKC independent in endothelial cells stimulated with IL-1 β or tumor necrosis factor- α (TNF α) (White and Lee, 1993). Furthermore, tyrosine kinase (PTK) inhibitors decreased IL-1 β -induced IL-8 production in endothelial cells (White and Lee, 1993), whereas in fibroblasts, protein kinase A

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(PKA) inhibitors decreased retinoic acid-induced IL-8 production (Zhang et al., 1992).

Transcription of the IL-8 gene is under the synergistic control of the transcription factors NF- κ B and NF-IL6 (Matsusaka et al., 1993). NF- κ B, whose inactive form is present in the cytoplasm, is activated upon phosphorylation and subsequent degradation of its inhibitory subunit I κ B. Subsequently, NF- κ B enters the nucleus (Baeuerle and Baltimore, 1988) and binds to its cognate sequence in the IL-8 promoter, resulting in enhanced IL-8 expression. Induction of NF- κ B by hydrogen peroxide, ionizing radiation, and LPS can be inhibited by PTK inhibitors (Geng et al., 1993; Schieven et al., 1993; Ishikawa et al., 1995), whereas phorbol ester and TNF α -induced NF- κ B is PKC dependent (Naumann and Scheidereidt, 1994). Less is known about the activation of preexisting NF-IL6. Phosphorylation of a threonine residue located just N-terminal to the DNA-binding domain by a ras-dependent MAP kinase has been suggested to be responsible for NF-IL6 activity (Nakajima et al., 1993).

ROIs have been increasingly studied for their involvement in signal transduction events (for review see Suzuki and Forman, 1997). For example, tyrosine kinase activity has been shown to be induced by H₂O₂ in combination with vanadate (Schieven et al., 1993) as well as by endogenously formed ROIs in neutrophils (Brumell et al., 1996). An ROI-induced increase in tyrosine phosphorylation could also be caused by a decreased tyrosine phosphatase activity, which contains oxidation-sensitive cysteine residues in its catalytic domain (Hecht and Zick, 1992). PKC activity could be sensitive to modulation by oxidants, because cysteine residues in the enzyme's regulatory and catalytic domain are susceptible to oxidation (Suzuki and Forman, 1997). In addition, oxidation of protein phosphatase 1 and 2A resulted in inhibition of these enzymes, which could result in PKC activation (for review see Suzuki and Forman, 1997). Rise in intracellular calcium levels ([Ca²⁺]_i), common to many signal transduction events, is also sensitive to redox regulation. Studies by us and others have shown that oxidant challenge increases [Ca²⁺]_i (Qu and Chen, 1995; Suzuki and Forman, 1997).

The cellular signaling steps involved in ozone-induced mediator production are not yet known. In this study, we investigated whether ozone-induced activation of the transcription factors NF- κ B and NF-IL6 as well as expression of IL-8 in a respiratory epithelial cell line is dependent on protein phosphorylation and sought to identify steps in the signaling cascade culminating in ozone-induced IL-8 production.

MATERIALS AND METHODS

Cell lines and cell culture

A549 cells, a human pulmonary type II epithelial-like cell line (American Type Cell Culture, Rockville, MD) was used throughout this study. This cell line was derived from a patient with alveolar cell carcinoma of the lung and retains some features of type II alveolar epithelial cells, including cytoplasmic multilamellar inclusion bodies (Lieber et al., 1976). A549 cells were cultured in F-12K media plus 10% fetal bovine serum (FBS) (Gibco BRL, Gaithersburg, MD) plus 1% penicillin and streptomycin (Gibco BRL).

In vitro exposure

A549 cells were grown on Vitrogen-coated (Collagen Corporation, Palo Alto, CA) Costar clear Transwells (25-mm diameter, 0.4- μ m pore size; Costar Corporation, Cambridge, MA) until a confluent monolayer was established (approximately 2×10^6 cells/well). A549 monolayers were exposed to either air or 0.1 ppm of ozone from the apical side at an air-liquid interface as described previously (Jaspers et al., 1997). Before exposure, cells were incubated for 1 h at 37°C with phenol red and FBS-free F12 nutrient mixture (Ham) containing one of the following: 1) the PKC inhibitors, Chelerythrine (20 μ M) or Calphostin C (1 μ M) (Calbiochem, San Diego, CA); 2) the PTK inhibitors, Genistein (10 μ M, Gibco BRL) or Herbimycin A (HA, 1 or 10 μ M, Drug Synthesis & Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD); 3) the PKA inhibitor, H89 (10 μ M and 100 μ M, Calbiochem), or 4) the hydroxyl radical scavenger dimethylthiourea (10 mM, DMTU, Sigma). Activity of Calphostin is light dependent, hence cells treated with Calphostin C were exposed to fluorescent light 1 h before exposure. For HA treatment, A549 monolayers were incubated with 1 μ M or 10 μ M HA 24 h and again 1 h before exposure based on studies described previously (Schieven et al., 1993). Genistein, Calphostin C, and HA were dissolved in dimethylsulfoxide (DMSO). In the experiments in which these inhibitors were used, cells were also treated with the most concentrated DMSO (0.5%, for HA) in parallel to the inhibitor treatment to serve as the vehicle control. The vehicle controls for the other inhibitors were all <0.1% DMSO and had no effect on ozone-induced IL-8 production (data not shown). Just before onset of exposure, the apical media, including the respective compounds, was aspirated, whereas 2 ml of media, including the various inhibitors, remained in the basolateral compartment to supply cells with nutrients and inhibitors during the exposure. Unexposed control cells remained covered with media for the exposure period. The exposure periods for the respective kinase assays were chosen based on kinetic studies, which indicated maximal kinase activities after 15- to 20-min ozone exposure. The 5-h exposure period for IL-8 mRNA and protein analysis were chosen based on results from previous experiments (Jaspers et al., 1997).

Electrophoretic mobility shift assay

Activation of cytosolic transcription factors and subsequent nuclear binding was assessed with the electrophoretic mobility shift assay. Nuclear factors for the mobility shift assay were prepared from A549 cells immediately after exposure as described (Jaspers et al., 1997). An oligonucleotide containing the NF- κ B binding site from the human IL-8 5' flanking region: 5'-ATCGTGGAATTTCTCTGACA-3' (BioSynthesis, Lewisville, TX) was labelled using [γ -³²P]ATP and T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The oligonucleotide containing the NF-IL6 consensus sequence (5'-TTCAACCTGTTCCGCAGTTTCTCGAGGAATC A-3') (Jaspers et al., 1997) was labelled using [α -³²P]CTP and Klenow enzyme (Boehringer Mannheim, Indianapolis, IN). Four micrograms of nuclear extract, 20,000 cpm of labelled probe, and 2 μ g of poly

dI/dC were mixed in a total volume of 20 μ l. The mixture was incubated on ice for 60 min, loaded on a 4% polyacrylamide gel, and subjected to electrophoresis at 20 mA for about 2 h. The gel was dried and autoradiographed on Kodak XAR5 film at 70°C for 13 days.

Analysis of RNA

The cells were exposed for 5 h to air/ozone as described above, and RNA was extracted 4 h after exposure using Trizol (Gibco BRL), according to the supplier's protocol. The amount of RNA was determined by A260/A280 spectrophotometric absorbance. Ten micrograms of RNA was loaded onto a 1.5% agarose gel and subjected to electrophoresis at 60 V for 4–5 h. The electrophoresed RNA was transferred onto membrane filters (Nytran, Schleicher & Schuell, Keene, NH) and fixed by ultraviolet crosslinking. Membrane-bound RNA was hybridized with cDNA for IL-8 (R&D Systems, Minneapolis, MN) and 28S (Clontech Laboratories, Inc., Palo Alto, CA) as described (Jaspers et al., 1997). IL-8 mRNA levels were normalized to 28S expression.

Analysis of PTK activity

Monolayers of A549 cells were exposed to air or 0.1 ppm of ozone as described above for 15 min. Cells were washed twice with phosphate buffered saline (PBS) (Gibco), and 250 ml of extraction buffer containing 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4, 50 mM β -glycerophosphate, 25 mM NaF, 0.1% Triton X-100, 150 mM NaCl, 20 mM ethyleneglycoltetraacetic acid (EGTA), 15 mM MgCl₂, 1 mM dithiothreitol, 25 μ g/ml leupeptin, and 25 μ g/ml aprotinin (all from Sigma) were added to each monolayer. Cells were scraped into the extraction buffer using a rubber policeman and were kept on ice. The cells were homogenized in a precooled Dounce homogenizer until no intact cells were visible under the microscope (about 20–30 strokes). The homogenates were incubated on ice for 30 min, and the cellular debris was removed by centrifugation at 14,000 rpm for 2 min. The supernatant was kept on ice until determination of tyrosine kinase activity using the protein tyrosine kinase assay system (Gibco). The tyrosine kinase assay was performed according to the supplier's instructions. Briefly, in the presence of [γ -³²P]ATP, 10 μ l of cell extract were incubated with either the substrate peptide RRSRC or a control solution without the substrate peptide. The substrate peptide is specific for tyrosine kinases and was derived from the amino acid sequence surrounding the phosphorylation site in pp60^{src} (Pike et al., 1982). The protein content of the supernatants was determined using the Bradford method (Bio-Rad, Hercules, CA), and tyrosine kinase activity was normalized to the protein content of the sample.

Analysis of PKA activity

A549 cell monolayers were exposed to air or 0.1 ppm of ozone for 15 min as described above. After exposure, cells were washed twice with PBS and PKA activity was analyzed using a protein kinase A assay system (Gibco) according to the supplier's protocol. Briefly, 250 μ l of extraction buffer [5 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris, pH 7.5] was added to the monolayer and A549 cells were scraped into the

extraction buffer using a rubber policeman. Cells were homogenized in a precooled Dounce homogenizer until no more intact cells were visible under the microscope (about 40–50 strokes). Cellular debris was removed by centrifugation at 14,000 rpm for 2 min. The supernatant was kept on ice until use in the PKA assay system and determination of protein content using the Bradford method (Bio-Rad). PKA activity was assayed as follows: 10 μ l of cell extract was mixed with either 10 μ l of diluent or 10 μ l of the PKA inhibitor PKI (Brumell et al., 1996; Lee et al., 1997) provided with the kit and incubated at room temperature for 20 min. After adding 10 μ l of [γ -³²P]ATP + PKA substrate "Kemptide" mixture to each sample, tubes were incubated at 30°C for 5 min. The difference in counts per minute of incorporated ³²P of the reaction mixtures containing diluent and inhibitor corresponds to PKA activity. All PKA activity values were normalized to the respective protein content.

Analysis of PKC activity

A549 cell monolayers were treated with 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA, 100 nM) or exposed to 0.1 ppm for 0, 5, 15, or 30 min as described above. After exposure, cells were washed twice with PBS and PKC activity was analyzed using a protein kinase C assay system (Gibco) according to the supplier's protocol. For comparison of PKC activity in the membrane and cytosolic fraction, A549 cells were scraped into 250 μ l of extraction buffer without detergent (20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β -mercaptoethanol, and 25 μ g/ml each aprotinin and leupeptin). The cells were homogenized (about 40–50 strokes), and the cellular debris containing the membrane fraction was pelleted by centrifugation at 14,000 rpm for 15 min at 4°C. The pellet was extracted again (homogenization using about 20–30 strokes) using 250 μ l of a detergent-containing extraction buffer (20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β -mercaptoethanol, 0.5% Triton X-100, 25 μ g/ml each aprotinin and leupeptin). The supernatants, containing the cytosolic and membrane fractions, were saved on ice until determination of protein content by Coomassie blue (Bio-Rad) or PKC analysis. This PKC assay system is based on the incorporation of [γ -³²P]ATP into Ac-MBP (4-14), a substrate peptide specific for PKC. Specificity is confirmed by addition of the potent inhibitor peptide PKC (19–36) to the reaction mixture. For analysis of PKC translocation to the cell membrane, the ratio of cytosolic to membrane PKC activity was computed.

Determination of IL-8

Immediately after the 5-h exposure period to air or ozone, all media were aspirated and 1 ml of fresh phenol red-free F-12 nutrient mixture was added to the apical and basolateral sides. Cells were cultured for an additional 4 h, and the conditioned culture media was collected for measurement of IL-8 levels. For analysis of TPA-induced IL-8 release, A549 cells were treated with 100 nM TPA for 30 min. As described above for air/ozone exposure, all media was aspirated after the TPA exposure and the cells were cultured in fresh phenol red-free F-12 nutrient mixture for another 2 h. Release of IL-8 by epithelial cells into the apical and basolateral compartments was assayed with a human IL-8

enzyme-linked immunosorbent assay kit (Endogen, Woburn, MA), according to the instructions given by the manufacturer. To assess total IL-8 released by A549 cells, the amounts of IL-8 secreted into the apical and basolateral compartments were combined and expressed as picograms of IL-8 per well.

Statistical analyses

Three Transwells per exposure and treatment group were used in this study. All data were analyzed relative to control. This analysis was done using a two-way analysis of variance to test for exposure or treatment effects. The Fisher post hoc test was used to determine whether the exposed groups were different from one another.

RESULTS

Effects of PTK, PKC, PKA, and ROI inhibitors on ozone-induced transcription of the IL-8 gene

To investigate the signaling steps involved in ozone-induced transcription of the IL-8 gene, we examined the effects of PTK, PKC, PKA, and ROI inhibitors on IL-8 mRNA levels in A549 cells exposed to either air or ozone. Pretreatment with the antioxidant DMTU decreased ozone-induced IL-8 mRNA, as shown in Figure 1A. Figure 1B shows the effect of the PTK inhibitor HA on ozone-induced expression of IL-8, illustrating that pretreatment with 10 μ M HA decreased ozone-induced IL-8 mRNA. This effect was not caused by the DMSO vehicle. Pretreatment with the PKA inhibitor H89 (100 μ M) decreased ozone-induced IL-8 mRNA levels, as shown in Figure 1C. Figure 1D shows the effect of the PKC inhibitors Calphostin C and Chelerythrine on ozone-induced IL-8 expression, indicating that pretreatment with either PKC inhibitor does not affect ozone-induced IL-8 mRNA levels. Figure 1E summarizes the densitometric readings of ozone-induced IL-8 mRNA in the presence of the various inhibitors. Ozone exposure significantly increases IL-8 mRNA levels compared with air-exposed cells. This effect was significantly decreased by pretreatment with DMTU, HA, and H89. The vehicle control for HA, 0.5% DMSO, as well as the two PKC inhibitors Calphostin C and Chelerythrine, failed to reduce ozone-induced IL-8 mRNA levels.

To ensure that treatment with the various inhibitors does not cause a nonspecific inhibitory effect on cellular metabolism but alters an ozone-induced signaling cascade, air-exposed cells were pretreated with PKC, PTK, and PKA inhibitors. Treatment with Calphostin C, HA, H89, or DMSO had no significant effect on air-induced IL-8 mRNA, as shown in the densitometric readings in Figure 2. As shown before in Figure 1E, treatment with HA and H89 significantly inhibited ozone-induced IL-8 mRNA, whereas treatment with Calphostin C failed to decrease IL-8 mRNA levels after ozone exposure. This finding suggests that treatment with Calphostin C, HA, H89, and DMSO has no effect on the general ability of A549 cells to synthesize IL-8.

Effects of PTK, PKC, PKA, and ROI inhibitors on ozone-induced IL-8 release

To investigate whether the above-described changes observed on ozone-induced IL-8 gene transcription are reflected in IL-8 release by A549 cells, the apical and basolateral conditioned media were analyzed for IL-8

content. As shown in Figure 3, the effects of the inhibitors on ozone-induced IL-8 production reflect the results seen in the analysis for IL-8 mRNA. The PTK inhibitors HA and Genistein both significantly decreased ozone-induced IL-8 production. The vehicle control for 10 μ M HA, 0.5% DMSO, also decreases IL-8 production. However, the vehicle effect is significantly less than that of 10 μ M HA-induced reduction, indicating that most of the effect is likely attributable to HA-mediated inhibition of PTK. The PKA inhibitor H89 and the antioxidant DMTU both significantly decreased ozone-induced IL-8 production. In contrast, the PKC inhibitors Calphostin C and Chelerythrine did not decrease ozone-induced IL-8 production.

To ensure that treatment with Calphostin C and Chelerythrine results in inhibition of PKC-dependent signaling, A549 cells were treated with TPA, a strong inducer of PKC activity, in the presence or absence of these two PKC inhibitors. As described before (Zang et al., 1992), TPA induces IL-8 release in A549 cells. As shown in Figure 4, treatment with either Calphostin C or Chelerythrine resulted in a significant decrease in TPA-induced IL-8 release.

Effects of PTK, PKA, and ROI inhibitors on ozone-induced transcription factors

To investigate whether the effects of the various inhibitors on ozone-induced IL-8 mRNA and protein levels are caused by a change in the transcriptional control of the IL-8 gene, we investigated the effects of PTK, PKA, and ROI inhibitors on ozone-induced NF- κ B and NF-IL6 activity. These two transcription factors have been shown to regulate synergistically the transcription of IL-8 (Matsusaka et al., 1993). Figure 5 shows the effects of the PTK inhibitors HA and Genistein, the PKA inhibitor H89, and the antioxidant DMTU on ozone-induced transcription factor activity. As reported by us (Jaspers et al., 1997), analysis of nuclear factors isolated from A549 immediately after exposure to either air or 0.1 ppm of ozone for 5 h increases the DNA-binding activities of NF- κ B and NF-IL6 (Fig. 5A and B, respectively). Pretreatment with the PTK inhibitors HA and Genistein as well as the antioxidant DMTU prevented the activation of NF- κ B and NF-IL6 after ozone exposure. The vehicle control for HA, 0.5% DMSO, had no effect on the ozone-induced DNA-binding activity of NF- κ B and NF-IL6. In addition, the PKA inhibitor H89 decreased ozone-induced DNA-binding activities of NF- κ B and NF-IL6. As reported by us previously, exposure to air had no effect on the DNA-binding activity of NF- κ B and NF-IL6 beyond the effects seen in unexposed/untreated control cells (Jaspers et al., 1997). Figure 5C and D show competition assays using an excess (50 \times) unlabelled NF- κ B and NF-IL6 oligonucleotide probes, demonstrating the specificity of the respective NF- κ B and NF-IL6 bands.

Effects of ozone exposure on PTK activity

To investigate the effects of ozone exposure on cellular tyrosine phosphorylation, we analyzed PTK activity in A549 cells after exposure to air or 0.1 ppm of ozone for 15 min. An effect of the PKA inhibitor or the antioxidant on ozone-induced PTK activity could indicate a possible sequence of signaling steps induced in A549 cells after ozone exposure. As shown in Figure 6, ozone-

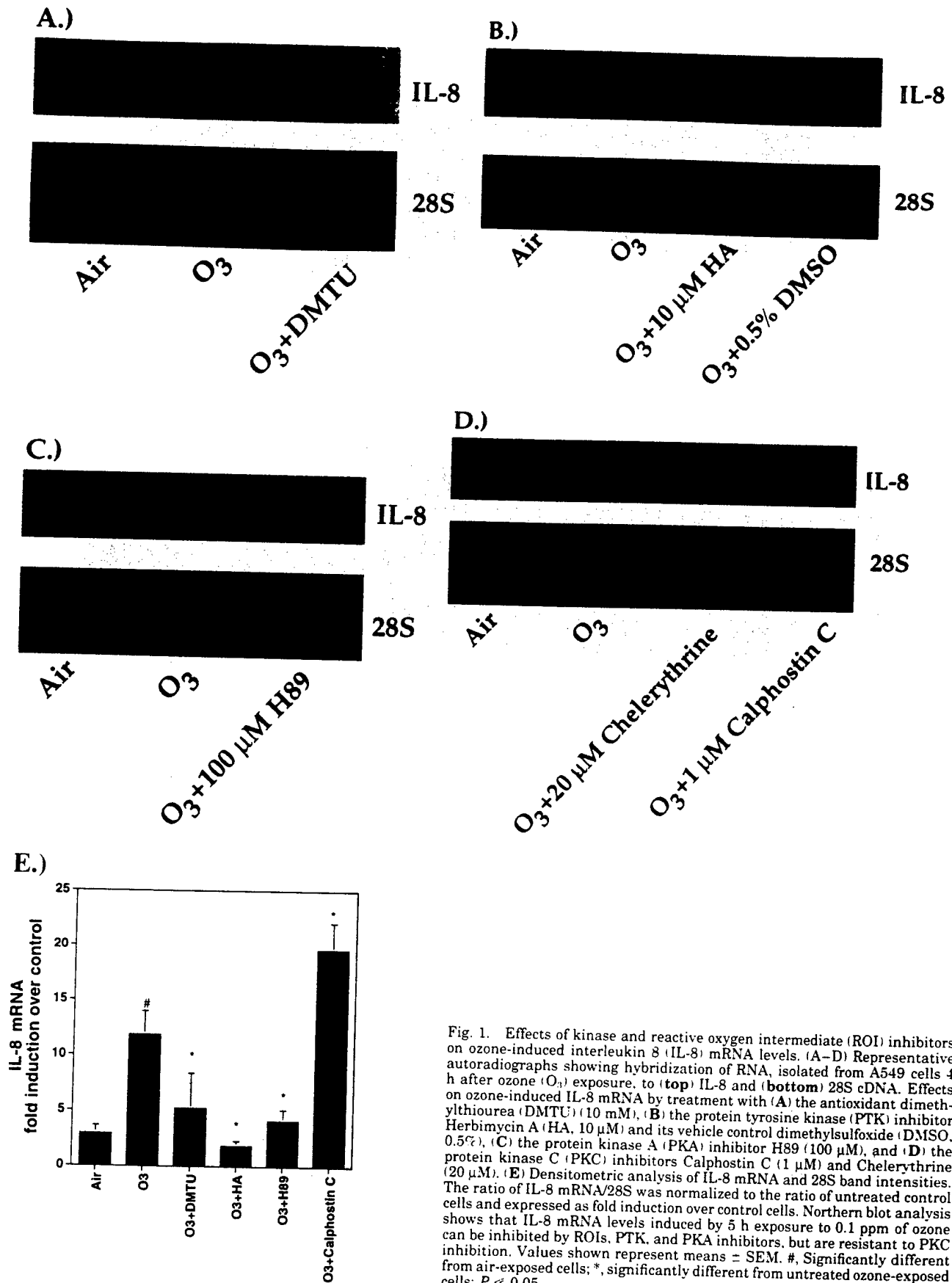


Fig. 1. Effects of kinase and reactive oxygen intermediate (ROI) inhibitors on ozone-induced interleukin 8 (IL-8) mRNA levels. (A-D) Representative autoradiographs showing hybridization of RNA, isolated from A549 cells 4 h after ozone (O₃) exposure, to (top) IL-8 and (bottom) 28S cDNA. Effects on ozone-induced IL-8 mRNA by treatment with (A) the antioxidant dimethylthiourea (DMTU) (10 mM), (B) the protein tyrosine kinase (PTK) inhibitor Herbimycin A (HA, 10 μ M) and its vehicle control dimethylsulfoxide (DMSO, 0.5%), (C) the protein kinase A (PKA) inhibitor H89 (100 μ M), and (D) the protein kinase C (PKC) inhibitors Calphostin C (1 μ M) and Chelerythrine (20 μ M). (E) Densitometric analysis of IL-8 mRNA and 28S band intensities. The ratio of IL-8 mRNA/28S was normalized to the ratio of untreated control cells and expressed as fold induction over control cells. Northern blot analysis shows that IL-8 mRNA levels induced by 5 h exposure to 0.1 ppm of ozone can be inhibited by ROIs, PTK, and PKA inhibitors, but are resistant to PKC inhibition. Values shown represent means \pm SEM. #, Significantly different from air-exposed cells; *, significantly different from untreated ozone-exposed cells; $P < 0.05$.

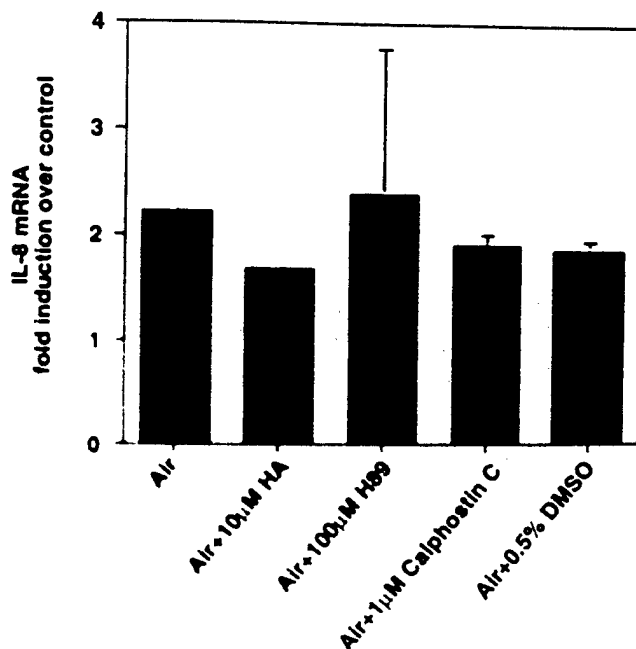


Fig. 2. Effects of protein kinase inhibitors on interleukin 8 (IL-8) mRNA levels in air-exposed cells. Densitometric analysis of IL-8 mRNA and 28S band intensities. The ratio of IL-8 mRNA/28S densitometric band analysis was normalized to the ratio of untreated control cells and expressed as fold induction over control cells. Northern blot analysis shows that IL-8 mRNA levels in air-exposed cells are resistant to treatment with protein tyrosine kinase, protein kinase A, and protein kinase C inhibitors. Values shown represent means \pm SEM. HA, Herbimycin A; DMSO, dimethylsulfoxide.

exposure significantly increased PTK activity in A549 cells. This response was dose-dependently inhibited by the PTK inhibitor HA. Moreover, pretreatment with DMTU reduced the ozone-induced PTK activity, although this effect was not statistically significant ($P = 0.07$). The PKA inhibitor H89 had no effect on ozone-induced PTK activity. The vehicle control for 10 μ M HA, 0.5% DMSO, slightly decreased ozone-induced PTK activity. However, the effect seen with HA (10 μ M) was significantly different from the DMSO control.

Effects of ozone exposure on PKA activity

Because the PKA inhibitor H89 inhibited ozone-induced transcription factor activity and IL-8 expression, we investigated the effects of ozone exposure on PKA activity in A549 cells. In addition, we examined the effects of treatment with the PTK inhibitor HA and the antioxidant DMTU on ozone-induced PKA activity. As mentioned above, an effect of these inhibitors on PKA activity would indicate a sequence of signaling steps induced by ozone-exposure in A549 cells. Figure 7 shows that exposure to ozone for 15 min significantly increases PKA activity compared with air-exposed cells. Treatment with H89 dose-dependently decreased ozone-induced PKA activity. DMTU and HA treatment significantly inhibited ozone-induced PKA activity. However, the inhibitory effect seen after treatment with 10 μ M HA could be due to the DMSO vehicle, because treatment with 0.5% DMSO also decreased ozone-induced PKA activity to a similar extent. Consid-

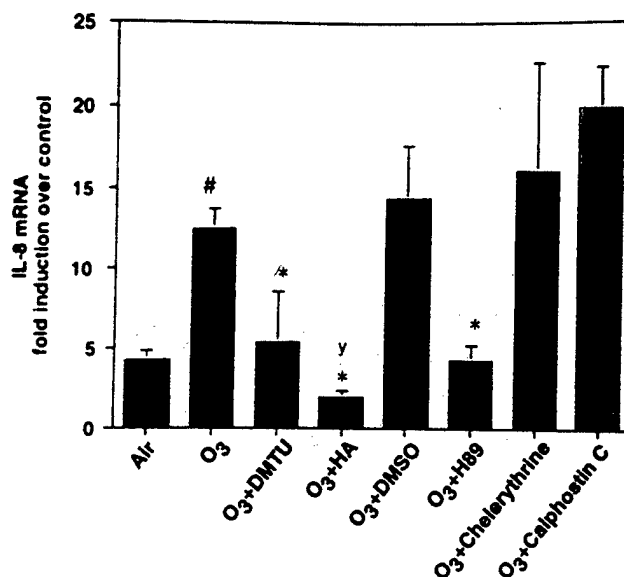


Fig. 3. Comparison of interleukin 8 (IL-8) production by ozone-exposed A549 cells treated with reactive oxygen intermediate, protein tyrosine kinase (PTK), protein kinase A (PKA), and protein kinase C (PKC) inhibitors. Conditioned media were collected 4 h after exposure to either air or 0.1 ppm of ozone (O₃). Analysis of IL-8 content shows that treatment with the antioxidant dimethylthiourea (DMTU), the PTK inhibitors Herbimycin A (HA) and Genistein, as well as the PKA inhibitor H89 decreases ozone-induced IL-8 production. The PKC inhibitor Calphostin C fails to decrease IL-8 production after ozone-exposure. #, Significantly different from air-exposed cells; *, significantly different from untreated ozone-exposed cells; y, significantly different from dimethylsulfoxide (DMSO) vehicle control; $P \leq 0.05$. Values shown represent means \pm SEM. (The error bar of IL-8 levels for the ozone + H89 samples was too small to be shown).

ering DMSO is also a strong antioxidant, this finding suggests a role for ROIs but not PTK in the ozone-induced PKA activity.

Effects of ozone exposure on PKC activity

Although the PKC inhibitors Calphostin C and Chelerythrine failed to inhibit ozone-induced IL-8 production, we examined the possibility that ozone exposure induces PKC activity, but is not essential for IL-8 production. Because PKC activity is typically accompanied by translocation of the enzyme activity to the membrane fraction of the cells, we analyzed the subcellular localization of PKC activity in ozone-exposed cells over time. As a positive control, A549 cells were stimulated with 100 nM TPA for 15 min, which has been shown to be a potent inducer of PKC activity in these cells (Dale et al., 1989). Figure 8 shows that ozone induced detectable, although not statistically significant, translocation of PKC activity from the cytosolic to the membrane fraction in ozone-exposed cells. On the other hand, stimulation with TPA significantly increases PKC activity translocation. This finding indicates that a brief ozone exposure does not significantly increase PKC activity in respiratory epithelial cells.

DISCUSSION

Despite the multitude of studies investigating the mechanisms of ozone toxicity, the cellular signals in-

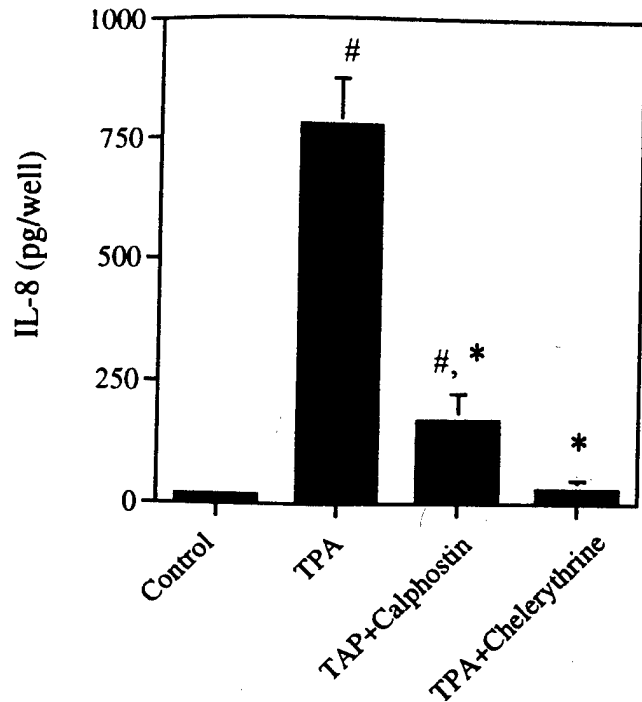


Fig. 4. Effect of protein kinase C (PKC) inhibitors on with 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced interleukin 8 (IL-8) release. A549 cells were stimulated with 100 nM TPA for 30 min in the presence of either Calphostin C (1 μ M) or Chelerythrine (20 μ M). Conditioned media were collected 2 h after TPA stimulation. Analysis of IL-8 content shows that stimulation with TPA significantly increases IL-8 release, which can be inhibited by treatment with either Calphostin C or Chelerythrine. #, Significantly different from control cells; *, significantly different from untreated TPA-stimulated cells; $P \leq 0.05$. Values shown represent means \pm SEM.

involved in ozone-induced inflammatory mediator release are not known. Previously, we demonstrated the ozone-induced activation of the transcription factors NF- κ B and NF-IL6 as well as production of IL-8 in respiratory epithelial cells. However, little is known about ozone-induced cell signaling beyond the initial oxidation at or near the cell membrane (Pryor et al., 1995). Therefore, we examined possible steps involved in the cellular cascade leading to ozone-induced IL-8 production in A549 cells, a respiratory epithelial cell line. Our results suggest that physiologic pathways mediate the ozone-induced IL-8 release by A549 cells. Specifically, we demonstrate that ozone-induced transcription factor activities and IL-8 expression are PTK and PKA dependent and PKC independent. These results illustrate that the ozone-induced signaling cascade mediating IL-8 production involves physiologic events similar to those previously shown to be used by other inflammatory stimulants (i.e., IL-1 β , TNF α) (Abreu-Martin et al., 1995).

Several different cell types secrete IL-8 in response to physiologic and pathologic agonists (Baggiolino and Clark-Lewis 1992). The cellular steps leading to IL-8 production have been studied to some extent and suggest that multiple pathways can mediate IL-8 production. For example, PTK-mediated IL-8 production was observed in response to TNF α and IL-1 β . This response

was, however, PKA independent (White and Lee, 1993; Abreu-Martin et al., 1995). PKA was shown to be involved in retinoic acid-induced IL-8 release, yet no indication for a role of PTK was discussed (Zhang et al., 1992). The results of this study suggest that ozone exposure induces IL-8 production by activating a signaling cascade involving both PTK and PKA.

The downstream substrates of protein kinase cascades involving PTK and PKA are often transcription factors (L'Allemain, 1994), which induce gene expression by facilitating the activity of RNA polymerase. Transcription of the IL-8 gene is controlled by the transcription factors NF- κ B and NF-IL6 (Matsusaka et al., 1993), which require Ser/Thr phosphorylation for their DNA-binding activity. Phosphorylation of two serine residues within the inhibitory subunit I κ B and its subsequent degradation allows NF- κ B to enter the nucleus and bind to its cognate DNA sequence (Chen et al., 1995). NF-IL6 requires phosphorylation of a threonine residue for translocation into the nucleus and DNA-binding activity (Nakajima et al., 1993). In the present study with A549 cells, we demonstrate that the ozone-induced DNA-binding activities of NF- κ B and NF-IL6 are PKA-dependent. This finding is in contrast to asbestos-induced NF- κ B and NF-IL6 activities in A549 cells, which were demonstrated to be PKC but not PKA dependent (Simeonova and Luster, 1996). Thus, in A549 cells, ozone-induced activation of transcription factors is mediated by different signaling events than asbestos fibers, even though asbestos, like ozone, is thought to exert its effects through the generation of oxidative stress (Simeonova and Luster, 1996).

As alluded to above, phosphorylation of tyrosine residues is not essential for the activation of these transcription factors. PTK-dependent kinase cascades have been shown to induce MAP kinase(s) in many systems (L'Allemain, 1994) that have been implicated in the regulation of NF- κ B and NF-IL6 DNA binding activities (Nakajima et al., 1993; Hirano et al., 1996). Thus, PTK-dependent MAP kinase activity could mediate ozone-induced NF- κ B and NF-IL6 activities. In contrast, the role of PKA in the MAP kinase cascade was originally described as inhibitory (Cook and McCormick, 1993). Recently, however, several studies reported the positive regulation of MAP kinases by PKA (Yarwood et al., 1996; Yee and Worley, 1997). Furthermore, the activated catalytic domain of PKA is able to translocate into the nucleus and directly phosphorylate and activate transcription factors (Comb et al., 1986). For example, phosphorylation of c-Rel by PKA has been suggested to increase the DNA-binding activity of NF- κ B (Lahdenpohja et al., 1996). Hence, PTK and PKA-dependent kinase cascades could be involved in the ozone-induced activation of NF- κ B and NF-IL6 by means of two different mechanisms: a) the direct phosphorylation of the transcription factors or b) the modulation of MAP kinase cascades, which subsequently would result in the phosphorylation and activation of NF- κ B and NF-IL6. More detailed studies investigating the role of MAP kinase(s) in ozone-exposed respiratory epithelial cells are necessary to establish a PTK and/or PKA-dependent activation of MAP kinase(s) in this model.

Previous studies indicated a role for ROIs in the activation of PTK (Schieven et al., 1993; Brumell et al., 1996). In addition, ROIs can affect tyrosine phosphory-

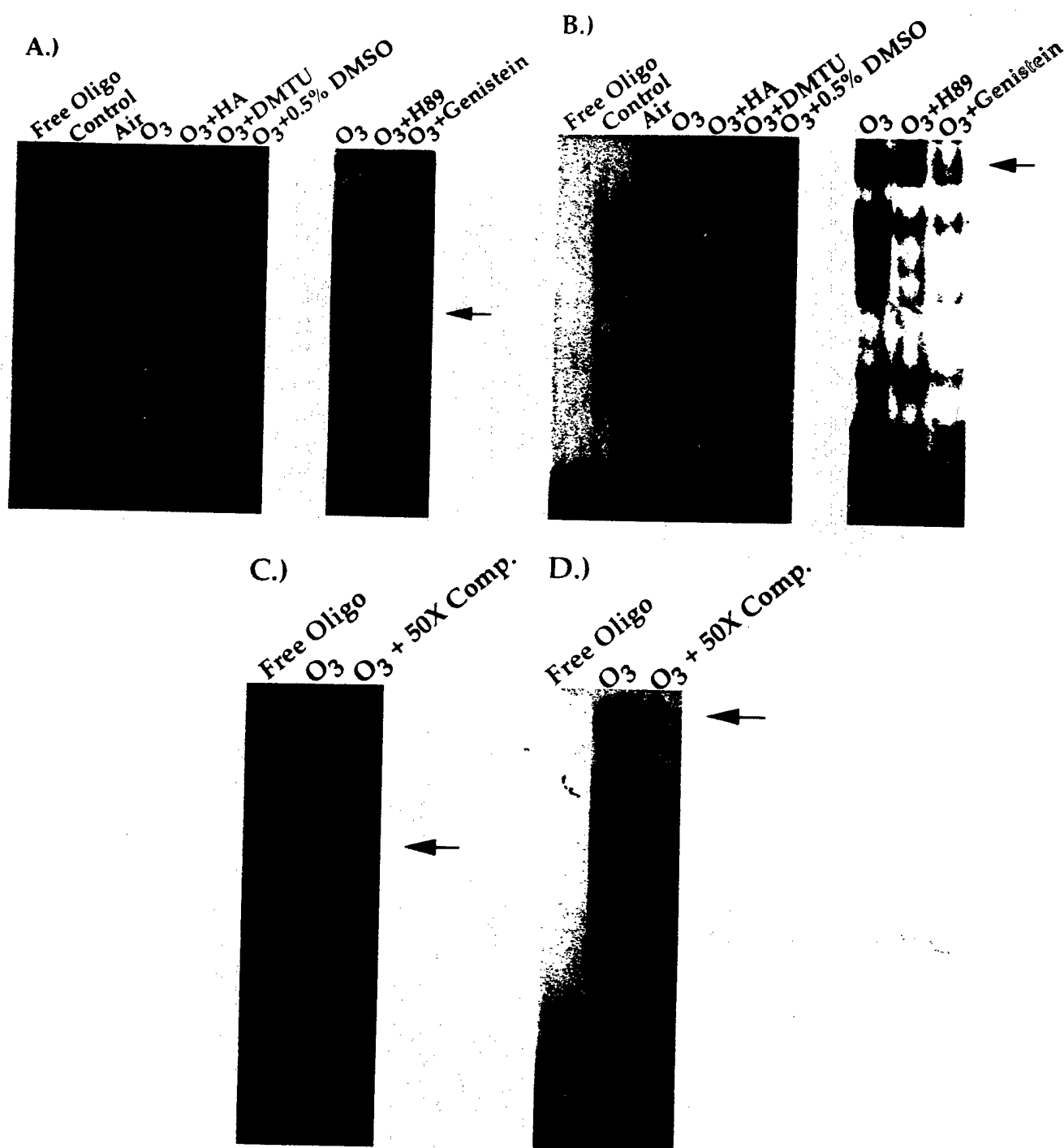


Fig. 5. Effects of reactive oxygen intermediate, protein tyrosine kinase (PTK), and protein kinase A (PKA) inhibitors on ozone (O₃)-induced transcription factor activities. Nuclear factors isolated from A549 cells immediately after 5 h exposure were analyzed for the effects of the PTK inhibitors Herbimycin A (HA, 10 μ M) and Genistein (100 μ M), the antioxidant dimethylthiourea (DMTU, 10 mM), and the PKA inhibitor H89 (100 μ M). Analysis of (A) NF- κ B and (B) NF-IL6 DNA-binding activity show that ozone-induced transcription factor activi-

ties can be inhibited by DMTU, HA, Genistein, and H89. Air exposure did not increase NF- κ B and NF-IL6 DNA-binding activity over basal levels seen in unexposed control cells. The vehicle for HA (0.5% dimethylsulfoxide (DMSO)) had no effect of ozone-induced DNA-binding activities. The arrows indicate the specific DNA-protein interaction band, which was identified by competition assays for (C) NF- κ B and (D) NF-IL6 by addition of excess (50 \times) unlabelled oligonucleotide probes.

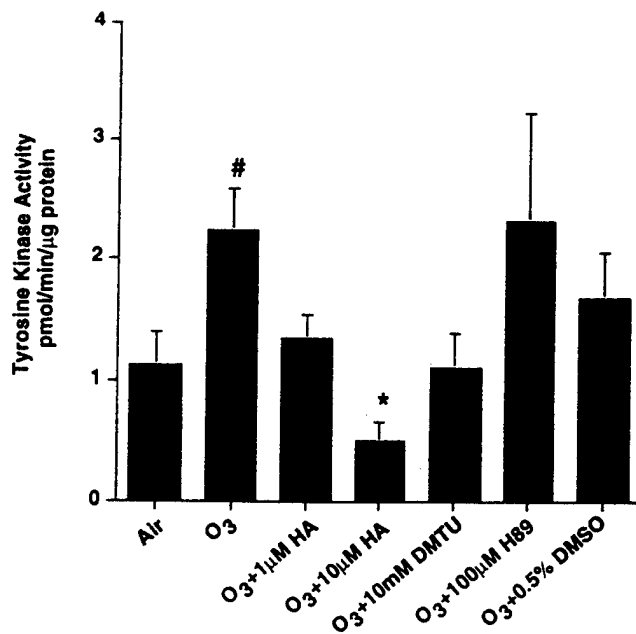


Fig. 6. Analysis of protein tyrosine kinase (PTK) activity in cells exposed to either air or 0.1 ppm of ozone (O₃) for 15 min. Extracts prepared from ozone-exposed cells treated with the PTK inhibitor Herbimycin A (HA, 1 μM and 10 μM), the antioxidant dimethylthiourea (DMTU) (10 mM), the protein kinase A inhibitor H89 (100 μM), and the vehicle control dimethylsulfoxide (DMSO, 0.5%) were analyzed for PTK activity. The assay measures the incorporation of [γ -³²P]ATP into a PTK-specific substrate peptide. Ozone exposure significantly increases PTK activity, which can be inhibited by HA. #, Significantly different from air-exposed cells; *, significantly different from untreated ozone-exposed cells; $P \leq 0.05$. Values shown represent means \pm SEM.

lation by means of decreasing tyrosine phosphatase activity. Oxidation of cysteine residues in the catalytic domain of tyrosine phosphatases have been shown to decrease their activity (Hecht and Zick, 1992). We demonstrate here that a low level of ozone induces PTK and PKA activity in a respiratory epithelial cell line within 15 min of exposure. In addition, activation of PTK and PKA appears to occur in parallel, because the specific kinase inhibitors failed to prevent the other kinase's activity. PTK and PKA activities were reduced by the antioxidant(s), indicating formation of ROIs to be a proximal step in the ozone-induced kinase activities. A role for ROIs in PKA activation is not yet clear. Whereas exposure of fibroblasts to hydrogen peroxide decreased PKA activity (Raynaud et al., 1997), oxidized lipids were shown to increase PKA activity (Parhami et al., 1995). Furthermore, lipid peroxidation, a common effect observed after ozone-exposure (Pryor et al., 1995), was shown to decrease phosphodiesterase activity, the enzyme mediating inactivation of PKA (Tyurina et al., 1993). Recent observations suggest that formation of LOPs rather than oxyradicals mediate ozone toxicity (Pryor et al., 1995). Thus, ozone-derived ROIs, lipid peroxidation, or LOPs may activate PTK and PKA in ozone-exposed respiratory epithelial cells.

The potential role of oxidation reactions in ozone-induced formation of IL-8 makes it difficult to use protein kinase inhibitors that must be dissolved in DMSO,

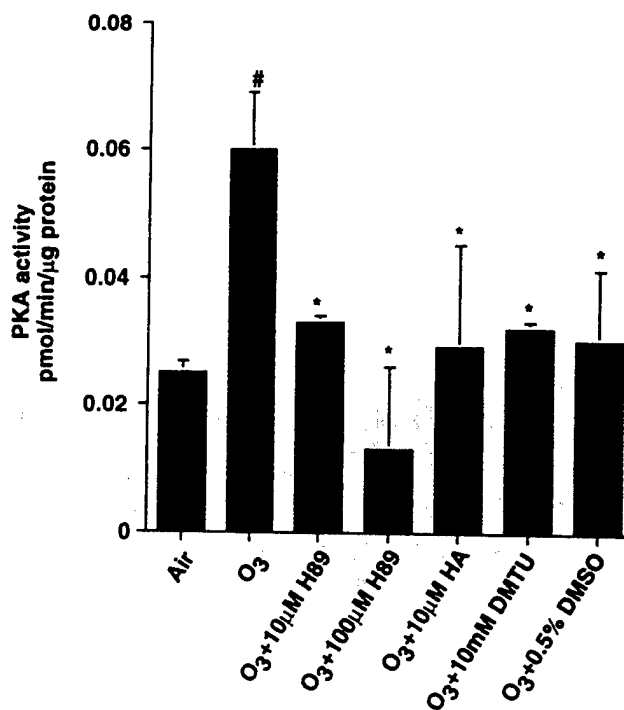


Fig. 7. Analysis of protein kinase A (PKA) activity in cells exposed to either air or 0.1 ppm of ozone (O₃) for 15 min. Extracts prepared from ozone-exposed cells treated with the PKA inhibitor H89 (10 μM and 100 μM), the protein tyrosine kinase inhibitor Herbimycin A (HA, 10 μM), the antioxidant dimethylthiourea (DMTU, 10 mM), and the vehicle control for Herbimycin A dimethylsulfoxide (DMSO, 0.5%) were analyzed for PKA activity. The assay measures the incorporation of [γ -³²P]ATP into a PKA-specific substrate peptide. Ozone-exposure significantly increases PKA activity, which was inhibited by H89, HA, DMTU, and DMSO. #, Significantly different from air-exposed cells; *, significantly different from untreated ozone-exposed cells; $P \leq 0.05$. Values shown represent means \pm SEM.

which by itself is a potent antioxidant and has been shown to reduce IL-8 levels (DeForge et al., 1993; Simeonova and Luster, 1996). Our results show that 0.5% DMSO, the most concentrated vehicle control used throughout the study, significantly reduced ozone-induced IL-8 protein levels as well as PKA activity. In the ozone-induced IL-8 protein levels, the effect induced by treatment with 10 μM HA was significantly different from its vehicle control 0.5% DMSO, indicating that the inhibition of PTK by HA mediates, at least partially, the reduction of ozone-induced IL-8 levels. On the other hand, ozone-induced PKA activity was equally inhibited by 10 μM HA and its vehicle control 0.5% DMSO, indicating that, here, the antioxidant activity of its vehicle DMSO mediated the inhibitory effect of HA on PKA activity. Thus, it is critical to evaluate the effects of the DMSO vehicle when studying signal transduction events induced by oxidants such as ozone.

In contrast to the above-described role of PTK and PKA, PKC inhibitors failed to decrease ozone-induced IL-8 production and PKC activity was not significantly elevated within 30 min of ozone exposure. Activation of PKC involves phospholipase C, which catalyzes the hydrolysis of phosphatidylinositol to inositol 1,4,5, tri-

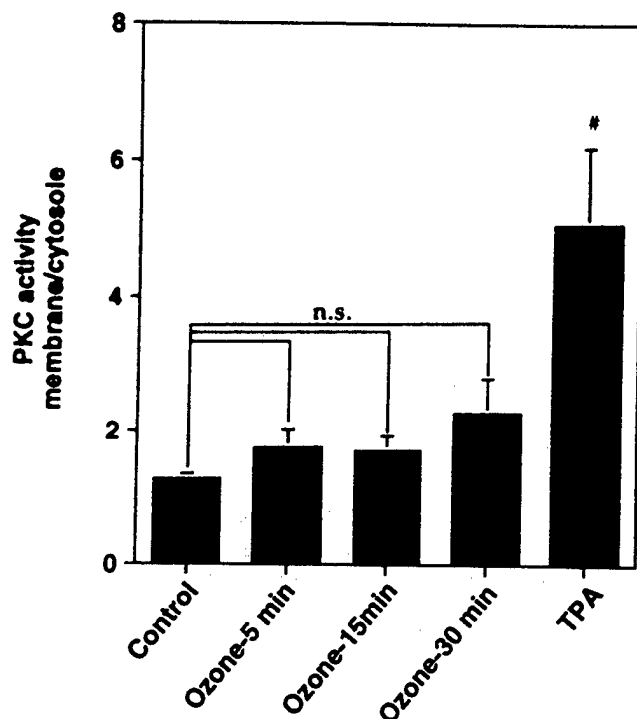


Fig. 8. Analysis of protein kinase C (PKC) activity translocation to the membrane fraction of the cell. A549 cells were treated with 100 nM with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) for 15 min or exposed to 0.1 ppm of ozone for 0, 5, 15, and 30 min and PKC activity was assayed separately in the cytosolic and membrane fraction. TPA treatment significantly induced PKC activity, whereas no significant movement of PKC activity from the cytoplasm to the membrane was seen within 30 min of ozone exposure. #, Significantly different from unexposed control cells. Values are expressed as the ratio between PKC activities in the membrane and cytosolic fractions and shown as means \pm SEM.

phosphate (IP_3) and diacylglycerol (DAG). DAG in turn activates PKC, whereas IP_3 increases $[Ca^{2+}]_i$ required for activation of Ca^{2+} -dependent PKC isotypes (Ryves et al., 1991; Asaoka et al., 1992). Previous studies in our laboratory demonstrated a rise in $[Ca^{2+}]_i$ upon ozone exposure (Qu and Chen, 1995). In addition, phospholipase C was activated in ozone exposed guinea pig tracheal epithelial cells (Wright et al., 1994). Our results show, however, that PKC phosphorylation does not mediate ozone-induced IL-8 production in a respiratory epithelial cell line. Two potent PKC inhibitors that are capable of significantly reducing TPA-induced IL-8 release in A549 cells failed to decrease ozone-induced IL-8 production. Furthermore, a brief ozone exposure failed to increase significantly translocation of PKC activity from the cytoplasm to the membrane fraction. Although prolonged ozone exposure may induce PKC activity and a PKC-dependent signaling cascade, our results suggest that in ozone-exposed A549 cells early signaling events mediating IL-8 production are PKC independent.

In conclusion, the results of our study indicate that in A549 cells, ozone-induced IL-8 production is PTK and PKA dependent and PKC independent. Furthermore, our data suggest that ozone-induced activation

of PKA and PTK occurs independent from each other and that both events follow an ozone-induced oxidation reaction. Exposure of these cells would lead to the formation of ROIs, lipid peroxides, or LOPs at or near the epithelial cell membrane (Pryor, 1995). These reaction products would mediate the response of respiratory epithelial cells to ozone by activating PTK and PKA. Activation of PTK and PKA would result in independent signaling cascades, possibly involving further steps such as the activation of MAP kinase(s) (L'Allemain, 1994), or directly modulate the activities of NF- κ B and NF-IL6 (Naumann and Scheidereidt, 1994). In contrast to NF-IL6, which could be directly phosphorylated by a MAP kinase (Nakajima et al., 1993), I κ B phosphorylation is likely to be mediated by a recently identified I κ B kinase complex (Zandi et al., 1997). However, other phosphorylation sites mediating activation of NF- κ B have also been identified (Naumann et al., 1994; Imbert et al., 1996) and might contribute to the ozone-induced NF- κ B activity. This study demonstrates activation of signaling cascades associated with the release of inflammatory mediators by respiratory epithelial cells exposed to ozone at levels encountered in urban area. Identification of signaling steps mediating toxicant-induced production of physiologic mediators, as demonstrated in this study, contributes to the understanding of toxicant-induced injury.

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